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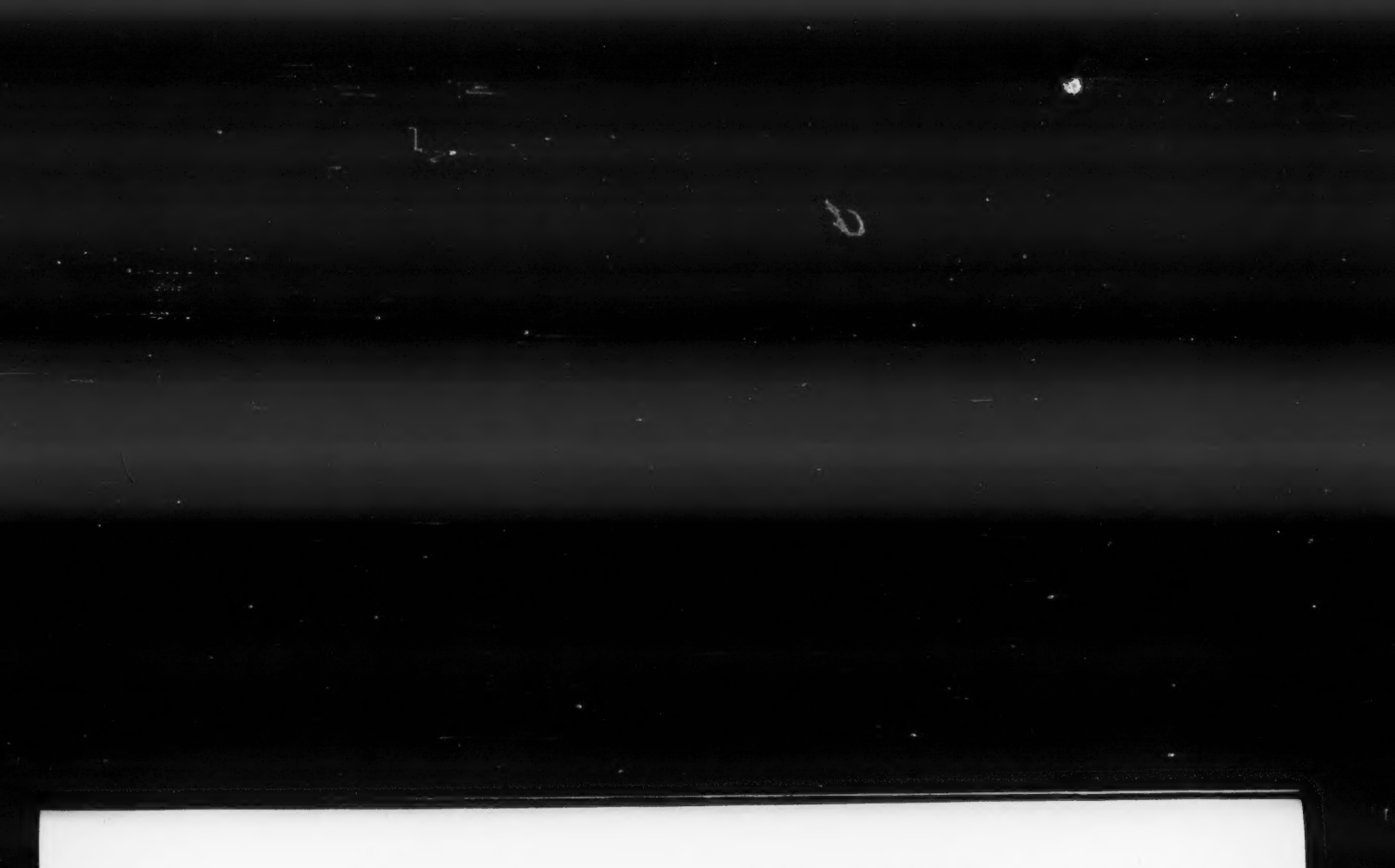

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Two Pathways from Brain Stem to Gamma Ventral Horn Cells.

By

RAGNAR GRANIT and BJÖRN HOLMGREN.[1]

Received 27 May, 1955.

It is known that the gamma impulses to the muscle spindles in many reflexes coincide with or even precede the motoneurone or alpha impulses (for a review, see GRANIT 1955). This means that such reflexes are accompanied by muscle spindle contractions with consequent excitation of their sense organs. Thus, although the spindle is in parallel with the muscle fibres, it is not unloaded (or passive) during the reflex but discharges impulses, serves as a measuring instrument, and through the myotatic reflex pathway influences the alpha ventral horn cells.

Now, when the gamma cells, and thus indirectly the spindles, are excited from the brain stem, one would expect this mechanism of precise alpha-gamma co-excitation to break down owing to the long conduction distance, unless there existed a pathway which in speed and efficiency compared favourably with the ones running the alpha motoneurones.

The experiments in which stimulation of the brain stem has been carried out (GRANIT and KAADA 1952, ELDRED, GRANIT and MERTON 1953) show this gamma-spindle mechanism of excitation to be highly efficient. Even the spindle impulses, which do not emerge until the original gamma-effect has traversed the muscular loop, may turn up in advance of alpha excitation and muscle contraction. The observation (GRANIT and KAADA, their Fig. 9) that

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when stimulating the mesencephalic tegmentum each shock may *drive* or excite a spindle impulse the way spindles can be driven by direct stimulation of gamma fibres (KUFFLER and HUNT 1952) suggests a fairly simple path.

The present experiments deal with measurements of conduction time in this pathway and with some attempts to interrupt it by acute spinal cord lesions. The measurements are given in terms of latent periods of the impulses evoked in single spindle afferents isolated in the dorsal roots and in a number of gamma fibres isolated in the ventral roots when stimulating the brain stem. For comparison with segmental reflexes, peripheral nerves have also been stimulated.

In view of the amount of work devoted to the gamma system one would have expected the chronological events to be well known. This, however, is not the case. For an idea of the peripheral time which has to be allotted to the loop (gamma ventral horn cell — muscle spindle end plate — sense organ excitation — dorsal root entry of afferent impulse) one has to turn to the records of KUFFLER and HUNT (1952) and HUNT and KUFFLER (1951) from which by estimation minimal values of 12—15 msec can be derived. When the same spindle is used as index both with a peripheral afferent stimulus and a shock to the brain stem, by subtraction of the respective central delays we have found the peripheral time to be quite constant. Thus it is actually possible, in comparing reflex times with central times, to use spindle afferents which so conveniently can be isolated in the afferent roots. It is, of course, necessary in such work to have myographic control.

Methods.

In the experiments 10 rabbits were used and 6 cats. The rabbits were anaesthetized with urethane (1.5 g/kg), the cats with chloralose (20 mg/kg) and nembutal (5 mg/kg). Operations were carried out under ether, sometimes supported by small doses of the short-lasting anaesthetic thiogenal (kindly supplied by the makers, Merck, Darmstadt).

The recording technique has been described in the papers by GRANIT and KAADA (1952) and ELDRED, GRANIT and MERTON (1953). The square wave pulses used by them were now modified by a transformer in the output circuit. In most experiments single shocks were employed, variable in duration, and applied at slow rates of repetition (once or twice a second) to the contralateral brain stem (HORSLEY-CLARKE approach), spinal roots, or peripheral nerves. Soleus-gastrocnemius muscle spindles were used.

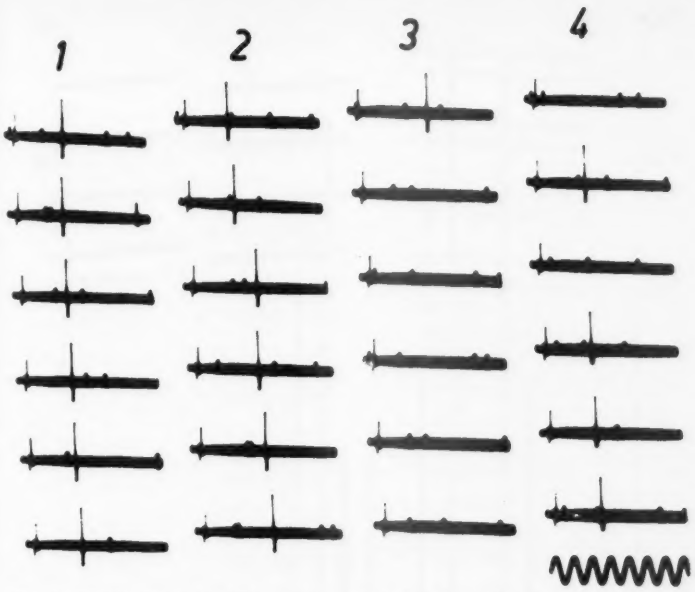

Fig. 1. *Rabbit. Single spindle response evoked from tegmentum of rabbit.* Gastrocnemius spindle. Stimulation of contralateral tegmentum. 1 msec shocks (see artifact) at intervals of 0.5 c/s. Time 100 c/s.

1, driving before myanesin. Latency 23—25 msec.
2, after myanesin, 20 mg/kg, injected intravenously during 15 seconds. Latency increase from 24 to 37 msec, and final disappearance of driven spikes in
3, 90 seconds later.
4, 2 minutes after injection. Driving normalized.

Results.

Spindle Latencies.

It is an advantage in work of this kind to have a muscle spindle which at light tension is relatively silent, in spite of intact gamma loop. This seemed easier to achieve with the rabbits than with cats. In Fig. 1 (1) stimulation of the mesencephalic tegmentum with 1 msec shocks at a rate of twice a second elicited a single spindle response to each shock. The latent period was fairly constant, 23—25 msec. A dose of 20 mg/kg of myanesin (BERGER and BRADLEY 1946) sufficed to delay it (2) and ultimately to remove the spike altogether (3). After a while the spindle response returned (4). This observation, originally made by GRANIT, HOLM-

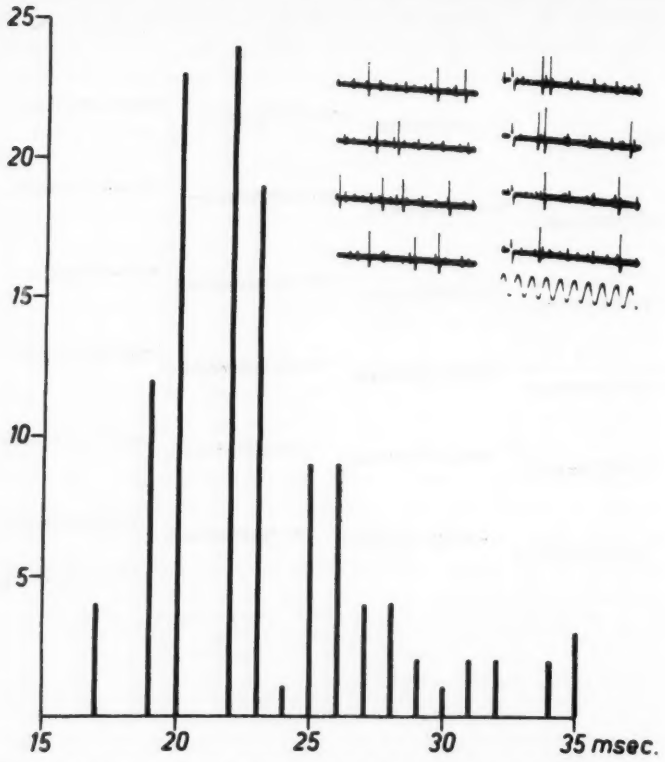

Fig. 2. *Cat. Latency of spindle responses evoked from the contralateral tegmentum* Horsley-Clarke coordinates AO, R3, H + 1 — H — 1. *Graph.* Abscissae: latency in msec. Ordinates: number of observations. About 120 values obtained by stimulating both at H + 1 and H — 1 plotted together. Stimulus duration: 2.6 and 2.1 msec respectively. Muscle tension: 150 g. *Inset.* On the left, basic "spontaneous" discharge. On the right, driving of spindle spike from H — 1. Time: 100 c/s.

GREN and LONGO (unpublished), suggested that there might exist a fast polysynaptic path from the mesencephalic tegmentum to the gamma ventral horn cells. One of us (R. G.) has since found that large doses of myanesin may depress the spindle discharge by a peripheral mechanism (anoxia?) but this effect chiefly engages the static or protracted discharge to stretch while the dynamic initial response is uninfluenced. Our original explanation that myanesin stops mesencephalic driving because of its well-known depressant effect on polysynaptic chains (HENNEMAN, KAPLAN

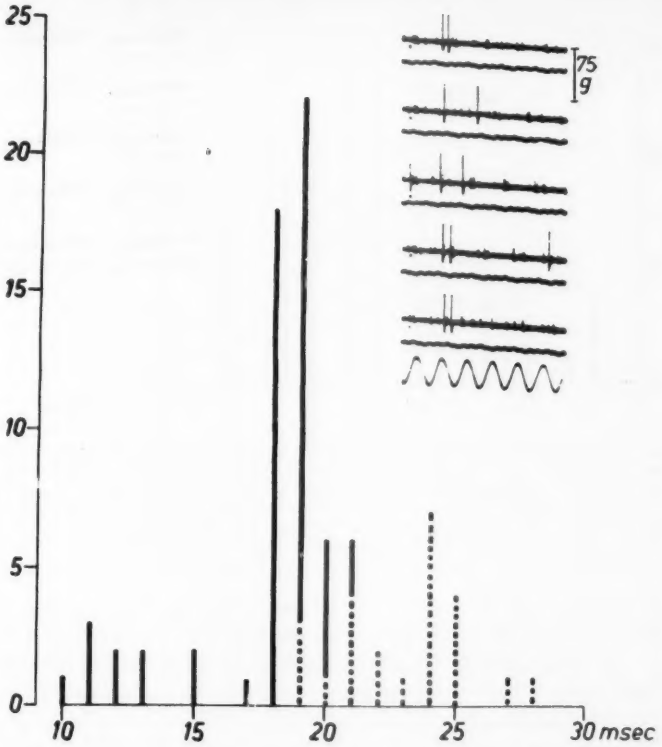

Fig. 3. *Driving of spindle from ipsilateral lateral popliteal nerve.* Same experiment as Fig. 2. Stimulus duration: 0.1 msec. Graph as in Fig. 2. Dotted lines correspond to latency of second spike. *Inset.* Spindle response and myogram. Myograph sensitivity alongside record. Muscle tension 180 g. Time: 100 c/s.

and UNNA 1949, KAADA 1950) must therefore be the correct one. In any case it led us to explore the latent periods in greater detail.

In cats there is generally a spindle discharge present when the muscle is at low initial tension, and even with slack muscle. An example is given in Fig. 2 (inset, left). In such cases it is necessary to take a large number of observations and treat them statistically. It is clear from Fig. 2 (inset, right) that the shocks to the brain stem actually drive the spindle. When the 120 observations were plotted (Fig. 2, main figure), it became possible to show that the driving had been very regular. The latent period of the first spike

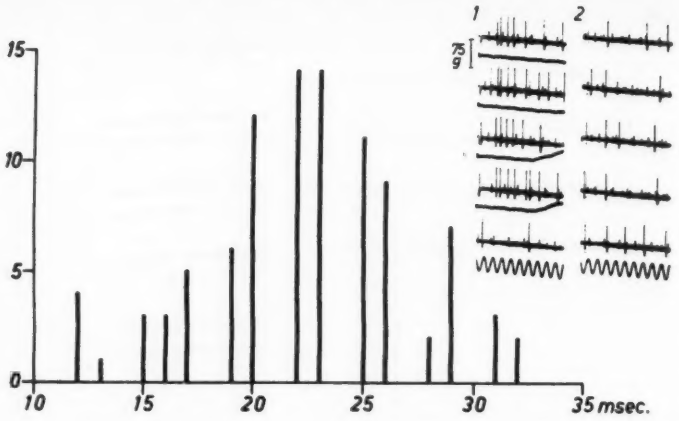

Fig. 4. *Cat. Crossed extensor reflex.* Stimulation of medial popliteal nerve. Stimulus duration 0.3 msec. *Inset: 1,* initial tension 380 g. Myograph sensitivity alongside record. Note, late contraction in 3rd and 4th sweep. Last sweep — no stimulation. *2,* initial tension 190 g, stimulus strength decreased. Shock artifacts marked by dot. Time: 100 c/s. Graph based on 120 values from the experiment with initial tension 190 g (2).

was around 20—23 msec. It is not uncommon to find a group of two or more spikes driven by the stimulus, often interfered with by the spontaneous discharge. Driving at slow rates from the brain stem hardly ever engages the alpha motoneurones which were myographically controlled.

Fig. 3, an ipsilateral reflex from the peroneal (lat. popliteal, JEFFERSON 1954) nerve, presents records in which the myograph response is included. Again there is a driven spike (inset), often double, and its latent period is 18—19 msec.

The ipsilateral latencies tend to be very precisely centered around a definite value. Contralateral gamma reflexes do not seem to activate a mechanism of corresponding temporal precision. Yet driving can be demonstrated in this case also. Fig. 4 refers to a crossed extensor reflex from the medial popliteal nerve. For the graph, records such as those on the right (inset) were used, muscle tension being 190 g. It was next raised to 380 g (inset, left) in order to obtain minimal muscular delay (KUFFLER and HUNT 1952). This shortened the latent period a little: from a minimum of 20 msec in the graph (at 190 g) to 18—19 msec. In this case, at the higher initial tension, a long repetitive discharge was driven

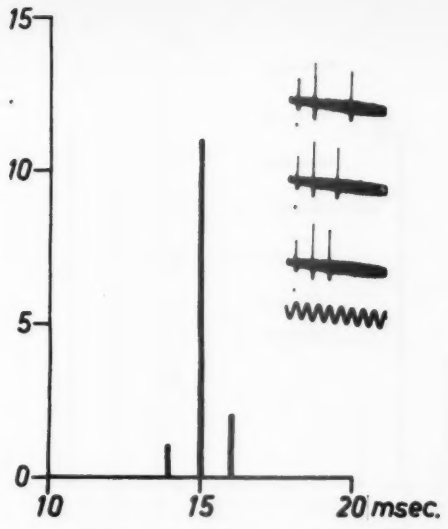

Fig. 5. *Cat. Ipsilateral reflex.* Gastrocnemius spindle in L7 dorsal rootlet. Stimulation of ipsilateral hamstring nerve, with 0.3 msec shocks. As Fig. 2. Time: 100 c/s.

and ultimately, after a latency of 60 msec, there followed a crossed extensor alpha reflex, as shown by the myogram (inset).

The ipsilateral hamstring nerves were found to give the shortest latent periods encountered in these experiments. The records and graph of Fig. 5 show it to be 15 msec. Three such experiments gave identical results.

Gamma Latencies.

Some experiments ended with dissection of filaments in the ventral roots, the brain stem electrode being left at the active site found. Gamma spikes were regularly found which were driven from this site. An example is given in Fig. 6. In the one case (6 A) a spindle response was driven with a latent period of 21 msec while the gamma latent period (B) was 9 msec. If these values be considered as representative (see Table 1), this would give a time of 12 msec for the peripheral loop, a value in good agreement with the minimal times obtainable from KUFFLER and HUNT's records (see above).

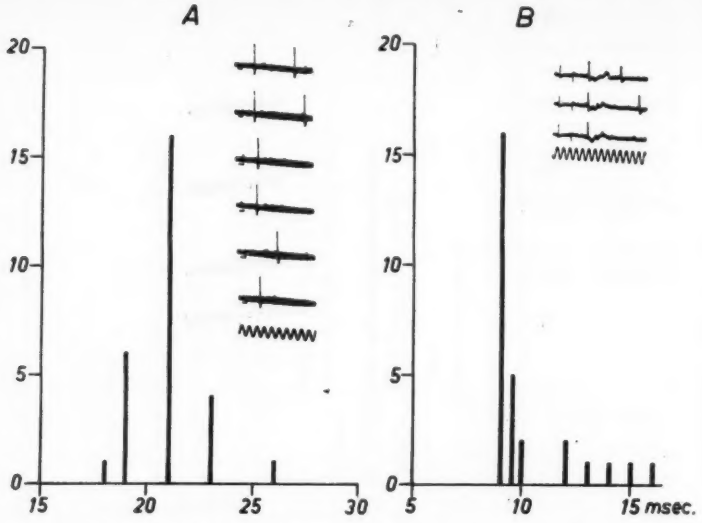

Fig. 6. *Cat. Tegmental driving of muscle spindle and gamma efferent.* Stimulation with 4 msec shocks.
A, recording from spindle afferent. Time: 100 c/s.
B, recording from gamma efferent in ventral L7. Time: 500 c/s.

In Fig. 7 A, a gamma spike driven from the ipsilateral lateral popliteal nerve had a latency of 5—6 msec. The latency of a driven spindle response was 18—19 msec giving thus a difference of 12—14 msec which can be allotted to the peripheral loop. Even if not directly determined, the latent period from the tegmentum to gamma fibre in this experiment would probably be around 8—9 msec. Table 1 shows that we have recorded tegmental gamma latencies from 7—12 msec. In Fig. 7 B the gamma latencies from the contralateral medial popliteal nerve was 8—9 msec. From the same place, at high initial tension, a spindle (Fig. 4) could be very effectively driven at a minimal latent period of 18—19 msec. If these values, too, be considered as representative, the minimum time spent in traversing the peripheral spindle loop would be of the order of 10 msec.

It is, of course, also possible to determine the 'loop time' directly by stimulating gamma fibres in efferent filaments and recording spindle impulses in the afferent roots in the usual way (KUFFLER and HUNT 1952). A disadvantage of this method is

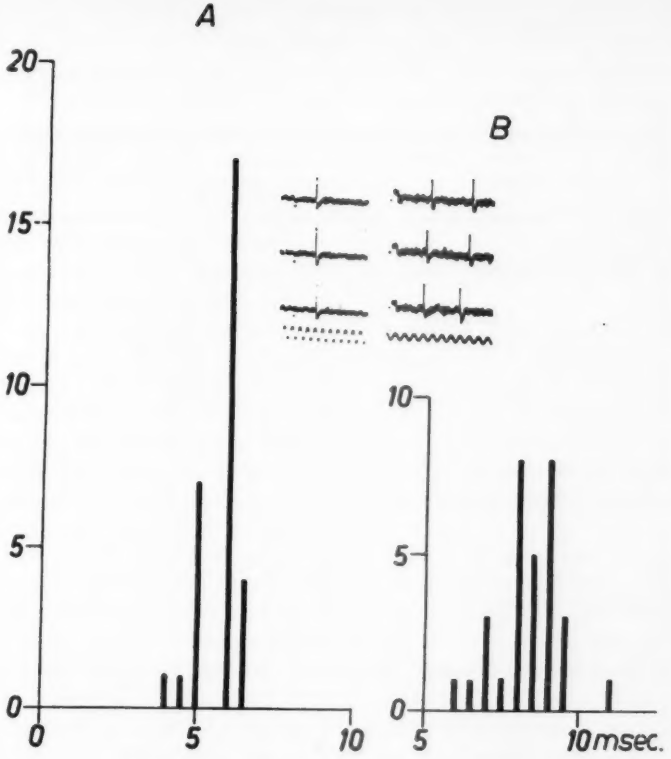

Fig. 7. *Cat. Driving of gamma efferent impulses.*
A, stimulation of ipsilateral popliteal nerve. Stimulus duration 0.1 msec (dots mark shock artifacts).
B, stimulation of contralateral popliteal nerve. 0.3 msec shocks. Time in both cases 500 c/s.

that one may not, with one or a few gamma fibres, obtain a spindle stimulation massive enough to give minimal times of activation. With an efferent filament dissected in this fashion we had a 'loop time' of 16—18 msec, with the other considerable variations, from 10 to 20 msec.

Table 1 summarises all our measurements, in so far as they gave precise latent periods and good driving. Cats and rabbits behaved very similarly. Assuming the 'loop time' in rabbits to be of the order found in cats both animals would be able to ac-

Table 1.

Latency of driven spikes.

Values are given in msec. When there is a clear preferential value one figure only is used. When the dispersion is greater, the distribution of the maxima is given. C, cats; R, rabbits.

From	Animal	To gamma efferents	To spindle afferents
Midbrain-pons, 3—4 mm lateral around Horsley-Clarke zero height	C 1		20—23
	C 2	9—9.5	21
	C 3	7—10	
	C 5	8—12	20—25
	C 6		20
	R 3		23—25
	R 5		25—30
	R 6		25
	R 8		26
	R 9		23
	R 10		20—25
Reticular substance. Medulla	C 3	6—7	
Ipsilateral lateral popliteal nerve	C 1	6—7	17—19
	C 2		26
	C 5		16—18
	C 6		20
	R 6		20—23
Ipsilateral hamstring nerve	C 2		15
	C 5		15
	C 6		15
Contralateral medial popliteal nerve	C 1	8—9	22—25 (150—190 g)
			18.5 (300 g)
	C 2		29
	C 5		26
L7 ventral rootlets	C 6		16—18
	C 6		10—20

tivate their efferent gamma fibres from the midbrain tegmentum within the brief time of 8—10 msec. This is a shorter time than the one (12 msec) necessary to activate alpha motoneurones by a short tetanus applied to the bulbar pyramids (LLOYD 1941, BROOKHART 1952) but slightly longer than the latency of lumbar ventral root discharges recorded when stimulating the reticular substance of the medulla (THULIN 1953) as read from his rather small-scale records.

The conduction distance from the mesencephalic tegmentum to the S1 root filament would be around 30 cm. However, the myanesin experiment suggested that the path might contain synapses.

Spinal Cord Lesions.

Our main new finding is that the midbrain possesses the fast and efficient spinal gamma pathway characterised above. On the other hand, repetitive stimulation of the brain stem at fast rates sets up a massive recruiting type of gamma activation which may outlast the stimulus for minutes (GRANIT and KAADA 1952). There are thus two modes of gamma activation, and so the question arises of whether they are being run in the same way through the same pathways.

In four rabbits and two cats acute lesions were made in the spinal cord while (i) driving and (ii) the effects of repetitive activation were followed on the screen and photographed. The cord was exposed from the region around L3, and from there downwards lesions were made with a scalpel in an attempt to interfere with the two modes of gamma activation. The spinal cords were embedded in celloidin and serial sections (50 μ) stained in thionin were used for reconstruction of the lesions.

Nearly all experiments were begun with complete contralateral cross sections which had no effect whatsoever on the driving of the muscle spindles from tegmental sites. Similarly it proved easy to show that incisions deep into the dorsal columns were without any influence. As incision followed incision downwards, leaving 3—4 mm intact spinal cord in between them so as to keep diffuse bleedings in check, it sometimes happened that the latent period lengthened a little and that driving required stronger shocks and a greater number of observations in order to show preference for one latency in the manner of the curves presented above. Mostly, however, it was possible to state that one particular incision, fairly deep, going laterally from the midline, did away with driving instantaneously (see analysis in Fig. 8).

The massive recruiting response of the spindles behaved very differently. It proved impossible to abolish it by any amount of criss-cross cutting into the spinal cord though very large ipsilateral incisions definitely diminished the effect. In many preparations it lasted until the very end, *i. e.* the final spinalization by one single section across the whole cord. This response thus represented a totally different physiological organization and pattern of behaviour, guarded by a safety margin approaching absolute non-vulnerability.

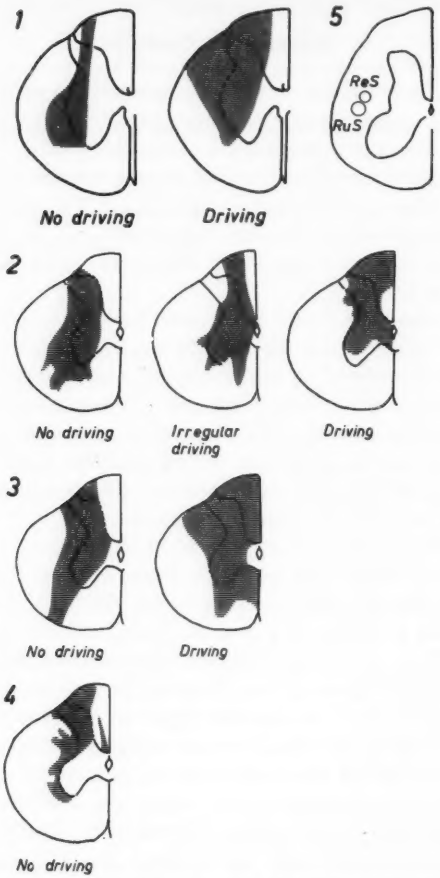

Fig. 8. Confluence areas of spinal cord incisions before and after the one that led to disappearance of driving, as described in text and marked below sections. *1—4* are four individual experiments of which *1*, cat, *3—4* rabbits. *5*, cross section of cat spinal cord, as drawn by PAPEZ (1929) with rubrospinal (RuS) and reticulospinal (ReS) tracts inserted.

Fully realizing the limitations of a technique employing acute incisions we were surprised, in studying the sections, to find fairly definite indications of where to look for the fast pathway running the gamma ventral horn cells from the brain stem in that particular region, L3 to L6. Fig. 8 which is self-explanatory, shows

tracings from spinal cord sections from those experiments which provided both decisive answers as well as good sections. The analysis suggests that far down in the spinal cord the critical point is in the lateral column at the level of the spinal canal. Further up in the spinal cord the path may well have taken a different course, particularly as we know that it has crossed one or several synapses. The schematic picture (5) is a cross section of the cat's spinal cord in which, according to PAPEZ (1929), the rubrospinal (RuS) and reticulospinal (ReS) tracts have been inserted. The former is well known to be particularly important in the cat. However, the pyramidal tract is not definitely excluded by these observations. NIEMER and MAGOUN (1947) found reticulo-spinal connections widely distributed in the lateral and ventral funiculi of the cord. However, they used repetitive stimulation and electromyography which makes differentiation of the kind attempted above impossible. Also, since the supraspinal gamma control was unknown at the time, it is impossible to say whether the motor effects obtained were run by the spindle loop, by intraspinal paths for the alpha ventral horn cells, or by both together. Nevertheless the lack of precise localization within the cord agrees with our results for the general repetitive mode of stimulation.

According to a personal communication from Prof. W. J. C. VERHAART "the only long uninterrupted thick fibres descending from the mesencephalon down into the spinal cord . . . in the cat are those of the rubro-, the tecto- and the interstitio-spinal systems. . . . Homogeneous tracts consisting of thick fibres do not occur within the reticular formation; the mentioned ones excepted."

It was hoped that the electrode sites in the brain stem, all of which were followed in 100 μ serial sections, would contribute to the localization. However, as long as the contralateral side was used and the electrodes kept some three mms or more outside the midline, there was no need for great precision in order to obtain driving. The histologically traced electrode sites spread all over the midbrain tegmentum into the posterior portion of the diencephalon at or below zero height on the HORSLEY-CLARKE coordinate system. Apparently, therefore, there is considerable convergence towards the site of origin of the fast path. There is, however, nothing to exclude that the final point of origin might be the red nucleus. Both rubro- and reticulospinal tracts are

crossed. The interstitio-spinal system is uncrossed (VERHAART). We did not succeed in driving spindles from the ipsilateral portion of the brain stem.

Discussion and Conclusions.

The experiments have led to the general conclusion that the tegmental influence on the gamma system controlling the muscle spindles is run by two different mechanisms, one fast, with the task of providing for immediate decisive action, the other slow, apparently dealing with semi-stationary tonic effects and also serving as a kind of volume control in general activation. The fast path crosses somewhere above the lumbar segments and may well above that region be the rubrospinal and/or tectospinal tract which cross just below their points of origin. A final decision requires localized stimulation within the cord and more precise lesions than is possible in acute experiments. The slow recruiting type of response from the midbrain stands massive lesions of the cord and so, despite grave spinal cord injuries, would always be expected to activate the spindles with consequent tonic reflex effects on the alpha motor system. There may well be pathological states in which something of this differentiation between a fast and precise mode of gamma activation and a slow tonic effect could be traced. With the fast path interrupted, the spindles, inasmuch as they are activated from high spinal or supraspinal sites, might be forced to passivity in alpha motor contractions run from corresponding regions, whereas their normal mode of activation is based on coexcitation of gamma and alpha motoneurones, muscle and muscle spindle contractions being thus coordinated. Dissociation of gamma from alpha activity has actually been observed with cerebellar lesions (GRANIT, HOLMGREN and MERTON 1955).

Inasmuch as voluntary control is exercised on muscles and their spindles simultaneously, it would also require the fast gamma path for quick and precise action. With this path the spindles would be able to act on the spinal cord while the pyramidal impulses are building up the necessary excitatory state in the segmental internuncial neurones.

Summary.

Spikes in single muscle spindle afferents from the dorsal roots (cat, rabbit) or individual gamma efferents from the ventral roots can be driven by shocks to the mesencephalic tegmentum or to afferent nerves (crossed as well as ipsilateral). The latent periods from the shock to the spike have been measured.

The experiments revealed the presence of a fast path from the mesencephalic tegmentum to the gamma ventral horn fibres with a latent period of only 8—9 msec (about 30 cm conduction distance). This path is crossed and, at the level of L3 to L6, can be destroyed by deep incisions into the lateral column of the spinal cord (see Fig. 8).

Another pathway is activated by repetitive stimulation of the mesencephalic tegmentum. Spikes cannot be driven over this route which elicits a recruiting protracted response. This path is widely distributed over the spinal cord and stands extensive criss-cross sectioning of the latter. Often the whole cord has to be divided for complete removal of the recruiting type of response.

It is suggested that the fast path serves spindle and motor co-operation in fast action and that the other path acts as a general 'volume control' of slow tonic effects.

In addition measurements are reported on gamma reflex times and the time required for the gamma effect to traverse the loop formed by gamma root efferent — muscle spindle — spindle root afferent.

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The Effect of Adrenaline on the Uptake of Glucose, Mannose and Fructose in the Rat Diaphragm.

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It has been shown by WALAAS and WALAAS (1950) that adrenaline inhibits the uptake of glucose in the diaphragm, an observation confirmed by SUTHERLAND (1952). This effect may explain the fact that adrenaline in vivo inhibits the assimilation of glucose in peripheral tissues (CORI, FISHER and CORI 1929, SOMOGYI 1950).

It has been assumed (SUTHERLAND 1952) that the effect of adrenaline on the uptake of glucose in the muscle depends on the accumulation of hexosemonophosphate which inhibits hexokinase reaction. An increase in the content of the hexosemonophosphate in muscle has been demonstrated both in vivo in rat muscle after the injection of adrenaline (CORI and CORI 1931) and in vitro in frog muscle on soaking with adrenaline (HEGNAUER and CORI 1934). That glucose-6-phosphate is a strong inhibitor of animal hexokinase, was originally demonstrated by WEIL-MALHERBE and BONE (1951) in the case of dissolved brain hexokinase and by CRANE and SOLS (1953) in particulate brain hexokinase and dissolved muscle hexokinase.

The present work concerns investigations on the inhibitory effect of adrenaline on the uptake of glucose, mannose and fructose in the diaphragm. Experiments to elucidate the mechanism of this adrenaline effect have been carried out. Some evidence

has been found that adrenaline may have a directly inhibiting effect on the uptake of glucose in the diaphragm, independent of an accumulation of hexosemonophosphate.

Experimental.

Experiments with the isolated diaphragm. Young male rats weighing 100—140 g were used in the experiments. The animals had fasted for 24 hours beforehand. 2 hemidiaphragms were incubated in 1 ml Krebs-Ringer-phosphate medium pH: 7.4 with 140 mg glucose per 100 ml medium, unless otherwise stated. Time of incubation 60 mins. at 37° C. The procedure is otherwise previously described in detail (WALAAS and WALAAS 1950, 1952).

The following reagents were used: 1-Adrenaline crist, codex. Rhône-Poulenc, Paris. The purity of the preparation was confirmed by spectrophotometric investigations according to LUND's (1949) method. Crystalline insulin from Nordisk Insulinlaboratorium of potency 27.4 units per mg was employed.

Glucose, "Bacto-dextrose", Difco, and mannose and fructose of special purity, from Hoffmann la Roche were used. The purity of hexose preparations was controlled by paper electrophoresis according to FOSTER (1952) and by an enzymatic method (STRECKER and KORKES 1952).[1] No impurities could be detected, in particular there was no glucose in the fructose preparation.

The concentration of hexose in the medium was determined by NELSON's method (1944). The fructose concentration was also determined by ROE's method (1934). The glycogen was determined according to WALAAS and WALAAS (1950), and lactic acid by BARKER and SUMMERSON's method (1941).

Hexosemonophosphate was analysed enzymatically with Warburg's Zwischenferment as given by SLEIN, CORI and CORI (1950). 200—300 mg diaphragm was extracted with 0.5 ml ice cold 3 per cent perchloric acid by grinding in a glass homogenizer. After neutralization with 2.0 ml 0.2 N sodium acetate determination of hexosemonophosphate was made in the filtrate.[2] An active Zwischenferment prepared by "Sigma" Chemical Company, and TPN (85 % degree of purity) also from "Sigma" Chemical Company were used. Inorganic phosphate was determined according to LOWRY and LOPEZ' (1946) method and phosphocreatine and ATP were determined after hydrolysis, according to FISKE and SUBBAROW's method (1925).

Experiments with homogenate and extract of diaphragm.

Adult rats weighing 200—300 g were used, and diaphragms from 4—5 animals were employed in each experiment. The diaphragms

[1] I should like to thank SÖREN LALAND, Ph. D. for having carried out these analyses.

[2] No inhibition of the Zwischenferment was exerted by $HClO_4$ or NaCN in concentrations present in the filtrate. (Experiments reported in Table 5).

were cut into small pieces and homogenised in a glass grinder of the Potter-Elvehjem type. The procedure was either carried out in isotonic KCl or isotonic sucrose, the pH was adjusted to approx. 8.0 by the addition of buffer. All procedures were carried out at + 1° C. The extract was prepared by centrifugation of homogenate in an International Refrigerated Centrifuge for 20 mins. at 15,000 × g.

0.5 ml 10 per cent homogenate or extract was incubated in a mixture containing (concentrations): 0.001 M glucose, 0.004 M ATP, 0.01 M $MgCl_2$, 0.053 M KF, 0.027 M $KHCO_3$. Total volume 3.0 ml.

Gassed out with 95 % N_2/5 % CO_2, pH was 8.0 and incubation carried out at 30° C for 20 mins. ATP in the form of Ba-salt was obtained from "Sigma" Chemical Company, converted to the potassium salt and used as such.

Results.

The inhibition of the uptake of glucose was examined with different concentrations of adrenaline in the medium (Fig. 1). The maximum effect was obtained with 1 μg of adrenaline and half the maximum effect with 0.20 μg per ml medium. A corresponding situation has been demonstrated in the case of the glycogenolytic effect of adrenaline on the diaphragm (E. WALAAS 1955).

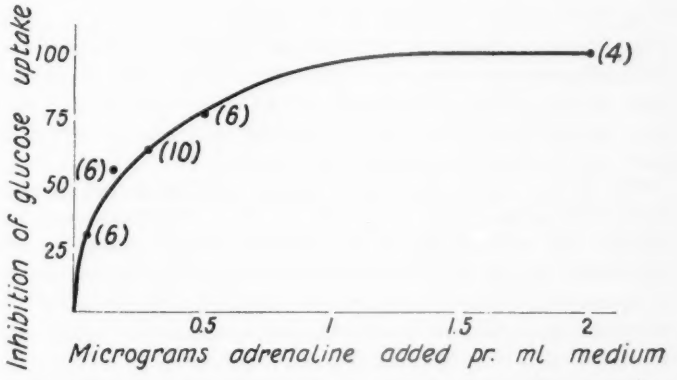

Fig. 1. Inhibition of the uptake of glucose in the isolated rat diaphragm with different concentrations of adrenaline. Incubation in 1 ml medium containing 140 mg glucose per 100 ml for 60 mins. at 37° C, 100 per cent O_2.

Inhibition of the uptake of glucose expressed in per cent of the maximum inhibition. Maximum inhibition: 0.8 mg per g per hour = 100. The points represent mean values.

Number of observations in ( ).

Table 1.

Inhibition of the uptake of glucose in the diaphragm by adrenaline with different concentrations of glucose in the medium. Incubation in 1 ml of medium. 2 micrograms adrenaline added in vitro. Incubation for 60 mins. at 37° C.

Experimental conditions	Glucose conc. in medium	Glucose uptake		Inhibition	
		Without adrenaline	With adrenaline	per cent	Statistical test
		mg per g per hour			
A. Aerobiosis	140 mg per 100 ml	[1] 2.5 ± 0.18 (13)	1.9 ± 0.12 (10)	24	t: 2.5 P: 0.05
	500 mg per 100 ml	5.7 ± 0.56 (5)	4.0 ± 0.45 (5)	30	t: 2.4 P: 0.05
B. NaCN added to the incubation medium	140 mg per 100 ml	2.55 ± 0.22 (14)	1.9 ± 0.16 (14)	25	t: 2.4 P: 0.04

A: 100 per cent O_2, *B:* 0.01 M NaCN in the medium. Number of observations in ( ).

[1] Standard error of mean.

As stated in Table 1, the effect of adrenaline was examined with different concentrations of glucose in the medium. It appears that the per cent of inhibition of the uptake of glucose was approximately the same at low (140 mg per 100 ml) and high (500 mg per 100 ml) glucose concentration.

The effect of adrenaline on the uptake of mannose and fructose in the diaphragm is given in Table 2. At a concentration of 140 mg per 100 ml of hexose in the medium, the ratio between the uptake of glucose and mannose was 1.0/0.6 while in the case of glucose and fructose the ratio between the uptakes was 1.0/0.5. A somewhat higher value for the uptake of mannose was found by Liebécq (1951), who reports a ratio of 1.0/0.8 between the uptakes of glucose and mannose. Adrenaline inhibits the uptake in the diaphragm of both mannose and fructose, but in the case of fructose a significant inhibition could only be detected with a high concentration of fructose in the medium. The effect of adrenaline on the uptake of mannose and fructose was in per cent approximately the same as for glucose.

Table 2.

Effect of adrenaline on the uptake of mannose and fructose in the isolated rat diaphragm. Incubation in 1 ml medium, added hexose in concentrations mentioned. 2 micrograms adrenaline added in vitro. Incubation for 60 mins. at 37° C, 100 per cent O_2.

Group	Hexose conc. in medium	Hexose uptake		Inhibition	
		Without adrenaline	With adrenaline	Per cent	Statistical test
		mg per g per hour			
Mannose[1]	140 mg per 100 ml	1.5 ± 0.18 (6)	0.8 ± 1.11 (6)	47	t: 3.3 P: 0.008
Fructose[1]	140 mg per 100 ml	1.25 ± 0.16 (10)	1.15 ± 0.16 (10)	8	t: 1.4 P: 0.2
Fructose[1]	500 mg per 100 ml	3.4 ± 0.42 (7)	2.3 ± 0.19 (7)	32	t: 2.4 P: 0.03
Fructose[2]	500 mg per 100 ml	2.0 ± 0.24 (5)	1.35 ± 0.20 (5)	32	t: 2.1 P: 0.07

In a number of experiments it has been investigated as to whether the adrenaline effect had any relation to the experimental conditions in vitro. Thus it was important to examine the hexokinase activity in the medium after the incubation, since ZIERLER, LEVY and ANDRES (1953) have demonstrated that glycogenolytic enzymes are liberated from the diaphragm into the medium during the incubation in vitro. However, own experiments[3] showed that the medium after 60 mins. incubation with the diaphragm had no certain hexokinase activity. The addition of adrenaline during the incubation did not have any influence.

The effect of adrenaline was not due to the liberation of reducing not fermentable substances from the diaphragm into the medium. The reduction value of the medium[4] fermented after

[1] The method of NELSON.

[2] The method of ROE.

[3] 0.4 ml medium was incubated in a mixture which contained 0.0012 M glucose 0.0025 M ATP, 0.01 M $MgCl_2$, 0.02 M KF, 0.03 M tris(hydroxymethyl)aminomethane buffer pH: 8.2. 20 mins. incubation at 30° C.

[4] Subsequent to the usual incubation of the diaphragm, the medium was fermented with baker's yeast. After precipitation with $ZnSO_4$ + $Ba(OH)_2$, the content of reducing substances was examined by Nelson's method.

Table 3.

The liberation of reducing substances from the diaphragm during the incubation. Incubation in 1 ml medium without glucose. 2 micrograms adrenaline added. Before the experiments the diaphragm had been washed 3 times with 25 ml ice cold medium. Incubation for 60 mins. at 37° C, 100 per cent O_2.

Group	Reducing compounds liberated to the medium mg per g per hour[1]	Glycogen breakdown in diaphragm mg per g per hour
Without adrenaline	0.05 ± 0.004 (6)	0 ± 0.13 (6)
With adrenaline ...	0.10 ± 0.02 (6)	0.5 ± 0.14 (6)

[1] Glucose equivalents.

the completion of incubation was zero both in controls and after the addition of adrenaline.

The possibility that the inhibition of the uptake of glucose is related to the glycogenolytic effect by the liberation of glucose has also been examined.

By degradation of glycogen free glucose may arise by α-1,6-glucosidase activity (CORI and LARNER 1951). On the incubation of the diaphragm in a glucose-free medium, a small amount of reducing substances was formed (Table 3). After the addition of adrenaline when there appears glycogenolysis in the diaphragm, the reduction value was somewhat greater, the increase corresponding to 10 % of the glycogen degradation. This however, constitutes only 2—3 % of the total glucose uptake when the diaphragm is incubated under usual conditions (Table 1), and is thus of negligible importance to the inhibition of the glucose uptake of adrenaline.

It has furthermore been investigated whether the effect of adrenaline has any relation to the content of sodium and potassium in the medium. It can be seen from Fig. 2 that the uptake of glucose in controls is inhibited by increasing concentrations of potassium in the medium. The effect of adrenaline in per cent however, is unchanged. The effect of adrenaline thus appears to have no special relation to the concentration of monovalent cations in the medium.

The following experiments had the purpose of investigating if adrenaline primarily inhibits the hexokinase reaction, independent of the glycogenolytic effect.

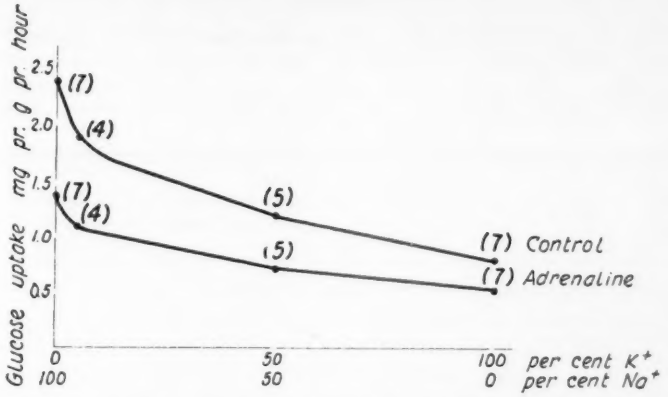

Fig. 2. Inhibition of the uptake of glucose in the rat diaphragm by adrenaline with different concentrations of sodium and potassium in the medium. The sum of sodium and potassium is kept constant (= 152 m. eq. per l), otherwise the composition of the medium is as usual. The points represent mean values. Number of observations in ( ).

As given in Tables 1 and 4, the effect of adrenaline on the diaphragm was examined after the blocking of the respiration by NaCN. In preliminary experiments it was shown that 0.01 M NaCN completely inhibited the O_2-consumption in the diaphragm and simultaneously a rapid degradation of the glycogen occurred. From Table 1 it is seen that adrenaline inhibited the glucose uptake[1] with a value of 25 % on the average, a value which is of marginal statistical significance (P: 0.04). Furthermore it is seen in balance experiments (Table 4) that the degradation of glycogen as well with as without adrenaline is of the same magnitude and almost complete. On the other hand production of lactic acid after the addition of adrenaline was inhibited with a value which corresponds to the inhibition of the glucose uptake.

The concentration of hexosemonophosphate in the diaphragm has also been examined. Under aerobic conditions, adrenaline gave an accumulation of hexosemonophosphate compared with the control values (Table 5). In the experiments where NaCN had been added to the medium, the concentration of hexose-

[1] NaCN reduced the values obtained in the method of Nelson by 5 %, apparently by cyanohydrin formation. A correction was therefore introduced.

Table 4.

The effect of adrenaline and insulin on the uptake of glucose, the breakdown of glycogen and the formation of lactic acid in the diaphragm when NaCN is added to the medium. Incubation in 1 ml medium containing 140 mg glucose per 100 ml. 0.01 M NaCN was always present. Time of incubation 60 mins. at 37° C.

Group	Glucose uptake	Glycogen breakdown	Lactic acid production	Balance per cent[1]
	mg per g per hour			
Without adrenaline	2.9 ± 0.27 (6)	1.6 ± 0.12 (6)	4.1 ± 0.25 (6)	91
With adrenaline ... (2 μg per ml medium)	2.1 ± 0.20 (6)	1.8 ± 0.19 (6)	3.6 ± 0.30 (6)	92
Without insulin ...	2.95 ± 0.30 (6)	1.0 ± 0.18 (6)	3.7 ± 0.28 (6)	94
With insulin ..... (0.1 unit per ml medium)	3.8 ± 0.39 (6)	1.0 ± 0.17 (6)	4.6 ± 0.15 (6)	96

[1] The formation of lactic acid in per cent of the uptake of glucose + the breakdown of glycogen.

Table 5.

The effect of adrenaline on the content of hexosemonophosphate in the isolated diaphragm.

Experimental conditions	Incubation time minutes	Hexosemonophosphate in the diaphragm			Statistical test
		Without adrenaline	With adrenaline	Difference	
		Concentration: Moles × 10^{-3}			
A. Aerobiosis	0	1.9 (3)			
	60	1.45 ± 0.1 (6)	1.9 ± 0.15 (6)	+ 0.45 (6)	t: 2.4 P: 0.04
B. NaCN Added to the medium	10	0.17 (3)	0.18 (3)	+ 0.01	
	30	0.18 (3)	0.16 (3)	— 0.02	
	60	0.09 (2)	0.09 (2)	0	

A. Incubation in 1 ml medium containing 140 mg glucose per 100 ml. 2 micrograms adrenaline added. Incubation at 37° C, 100 per cent O_2.

B. Incubation in 1 ml medium containing 140 mg glucose per 100 ml. NaCN was present in a concentration of 0.01 M. 2 micrograms adrenaline were added. Incubation at 37° C.

Table 6.

The water content in the diaphragm after the addition of adrenaline in vitro. Experimental conditions as described in Table 5.

Group	Without adrenaline	With adrenaline
	Per cent H_2O in the diaphragm	
A. Aerobiosis	75.7 ± 0.6 (8)	75.4 ± 0.4 (8)
B. NaCN added to the medium	79.1 ± 0.4 (7)	79.1 ± 0.3 (7)

monophosphate was rapidly reduced to 1/10 of the aerobic value and adrenaline did not here influence the concentration. In these latter experiments, adrenaline thus inhibited the glucose uptake in the diaphragm (Table 1) without the occurrence of any accumulation of hexosemonophosphate.

The possibility that adrenaline produces changes of water content in the diaphragm has also been investigated. These experiments have produced negative results. The water content in the diaphragm with and without adrenaline was of the same magnitude (Table 6). This was shown both by aerobic conditions and when NaCN was added to the medium. The water content after the addition of NaCN was somewhat higher than that under aerobic conditions.

As shown in Table 4, the effect of insulin on the glucose uptake was also investigated after the addition of NaCN to the medium. A definite stimulation of the glucose uptake by insulin was shown under these conditions, with a corresponding increase in lactic acid production. The effect of insulin on the glucose uptake was however only 50 % of the effect of insulin under aerobic conditions. These experiments confirm previous experiments on the effect of insulin under anaerobic conditions (Walaas and Walaas 1952).

Experiments on the antagonistic effect of adrenaline and insulin on the glucose uptake in the diaphragm were also carried out. Rieser, Wecke and Ther (1949), have shown that when insulin is added to the medium at the same time as adrenaline during incubation, the glycogenolytic effect of adrenaline on the diaphragm is abolished. In the following experiments, a somewhat different technique is employed. The experiment recorded in

Table 7.

The antagonistic effect of adrenaline and insulin on the uptake of glucose in the isolated diaphragm. Incubation in 1 ml medium containing 140 mg glucose per 100 ml. Incubation for 60 mins. at 37° C, 100 per cent O_2.

Group I		Group II		Group III	
Without adrenaline	With adrenaline	With adrenaline	With adrenaline + insulin	With insulin	With insulin + adrenaline
Glucose uptake mg per g per hour					
1.7 (3)	1.3 (3)	1.6 ± 0.20 (11)	2.3 ± 0.23 (11)	5.2 ± 0.32 (6)	2.8 ± 0.32 (6)

Group I.

The diaphragm was pre-exposed to the medium without and with a concentration of 2.7×10^{-4} M adrenaline in the medium at 25° C. Washed twice with 25 ml of incubation medium. Adrenaline was not added to the medium at the final incubation.

Group II.

The diaphragm was pre-exposed to the medium to which was added adrenaline in a concentration of 5.5×10^{-4} M for 5 mins. at 25° C. Washed twice with 25 ml of medium. The final incubation was carried out with and without the addition of 1 unit insulin per ml.

Statistical test: t: 2.3, P: 0.03.

Group III.

The diaphragm was pre-exposed to the medium containing 1 unit insulin per 5 ml for 5 mins. at 25° C. Washed twice with 25 ml of medium. The final incubation was carried out with and without the addition of 2 micrograms adrenaline per ml.

Statistical test: t: 5.4, $P < 0.001$.

Table 7, shows that when the diaphragm is pre-exposed to adrenaline an inhibition of the glucose uptake occurs. Considerably higher adrenaline concentration is however required and the effect is only 50 per cent of the effect found when adrenaline is present during incubation. Correspondingly, a slight glycogenolytic effect is shown (STADIE, HAUGAARD and MARSH 1951, WALAAS 1955), by the pre-exposure of the diaphragm to adrenaline. On the other hand, when the diaphragm is pre-exposed to insulin, quantitatively the same metabolic effect is produced as when insulin is added during the actual incubation.

From Table 7 it appears that insulin has an antagonistic effect on the glucose uptake in the diaphragm after the pre-exposure to adrenaline. In the same manner, the stimulation of the glucose uptake after pre-exposure to insulin will be abolished when the adrenaline is added during incubation. With the employed hor-

Table 8.

The effect of adrenaline on the content of acid soluble phosphorous compounds in the isolated diaphragm. Incubation under aerobic conditions as described in Table 1.

Group	Inorganic P	A T P Acid-labile P	Phospho-creatine P
	mg per g diaphragm		
Without adrenaline	0.29 ± 0.01 (6)	0.16 ± 0.02 (6)	0.31 ± 0.02 (6)
With adrenaline	0.29 ± 0.01 (6)	0.18 ± 0.02 (6)	0.26 ± 0.02 (6)

mone concentrations, the glucose uptake is affected in such a way that the values revert to the normal level. The experiments indicate that the glucose assimilation in muscle is directly regulated peripherally by insulin and adrenaline, while the quantitative proportions of the antagonistic effect have not been further examined.

The experiments carried out suggest that adrenaline has a primary effect on the hexokinase reaction in the diaphragm. Investigations on the effect of adrenaline on the concentration of ATP in the diaphragm and on the hexokinase activity in an homogenate of the diaphragm have therefore been carried out. A definite effect of adrenaline on the concentration of labile phosphate esters in the diaphragm could not be detected (Table 8). In particular, adrenaline did not affect the concentration of ATP, this has also previously been shown in the case of insulin (WALAAS and WALAAS 1952). This fact indicates that adrenaline does not inhibit the generation of energy-rich phosphate compounds in the diaphragm, such as does 2,4-dinitrophenol (JERVELL, WALAAS and WALAAS, 1955). It has also been demonstrated in experiments using homogenate of diaphragm that adrenaline in concentrations up to 3.6×10^{-4} M did not influence the liberation of inorganic phosphate from ATP.[1]

The effect of adrenaline on the hexokinase in homogenate and extract has further been investigated (Table 9). In a number of

[1] The following system has been used: 0.0022 M ATP, 0.01 M $MgCl_2$, 0.053 M KF, 0.03 M Tris(hydroxymethyl)aminomethane buffer, pH: 8.2. Diaphragm was homogenised in 0.25 M sucrose and 50 mg equivalent diaphragm added. Incubation for 20 mins. at 30° C.

Table 9.

The effect of adrenaline on the phosphorylation of glucose in homogenate and extract of rat diaphragm.

Group	Experiment number	Adrenaline Molar concentration	A T P Molar concentration	Glucose phosphorylation µg glucose	Inhibition µg glucose	Inhibition per cent
Homogenate[1]	1	0	3×10^{-3}	242		
		1×10^{-4}	3×10^{-3}	242	0	0
		1×10^{-3}	3×10^{-3}	209	33	14
	2	0	3×10^{-3}	403		
		5×10^{-4}	3×10^{-3}	408		
		1×10^{-3}	3×10^{-3}	379	24	6
	3	0	3×10^{-3}	233		
		5×10^{-4}	3×10^{-3}	217	16	7
	4	0	1.5×10^{-3}	125		
		5×10^{-4}	1.5×10^{-3}	110	15	12
	5	0	3×10^{-3}	311		
		5×10^{-4}	3×10^{-3}	312	0	0
	6	0	1.5×10^{-3}	147		
		5×10^{-4}	1.5×10^{-3}	146	0	0
	7	0	8×10^{-4}	26		
		5×10^{-4}	8×10^{-3}	13	13	50
Extract[2]		0	2×10^{-4}	376		
		5×10^{-4}	2×10^{-3}	350	26	7
		1×10^{-3}	2×10^{-3}	356	20	5
		0	4×10^{-3}	466		
		5×10^{-4}	4×10^{-3}	454	12	3
		1×10^{-3}	4×10^{-3}	447	19	4

experiments a slight inhibition of hexokinase on the addition of adrenaline has been detected, with values of inhibition ranging from 3—14 per cent. 10^{-3} M adrenaline[3] gave inhibition in all experiments, while the effect of 5×10^{-4} M did not give completely reproducible results. The inhibition by adrenaline was

[1] Homogenization of the diaphragm in 0.154 M KCl + 0.15 M $KHCO_3$, pH: 7.8.

[2] Homogenization of the diaphragm in 0.25 M sucrose + 0.01 M "Tris"-buffer, pH: 8.0.

[3] Spectrophotometric examination of the incubation medium after the experiment, showed that no oxidation of adrenaline to adrenochrome had occurred.

independent of the concentration of ATP in the system. The effect on the hexokinase was thus weaker and not so constant as the inhibition of the glucose uptake by adrenaline in the surviving diaphragm.

Discussion.

Earlier work has established that the uptake of glucose in the rat diaphragm is under hormonal control by insulin and adrenaline. This also occurs when mannose is the substrate. Thus insulin increases the uptake of mannose (LIEBÉCQ 1951) while inhibition of mannose uptake by adrenaline has been shown in the present work.

Contradictionary results have been obtained as far as the effect by insulin on fructose metabolism in vitro is concerned. In experiments on liver slices BAKER, CHAIKOFF and SHUSDIK (1952) reported that insulin was without any effect on the metabolism of fructose. On the other hand MIRSKY, HAFT and PERISUTTI (1953) observed significant stimulation of fructose uptake in the rat diaphragm by insulin. The fact that fructose is metabolized by different metabolic pathways in muscle and liver (HERS and KUSAKA 1953) may be of importance.

The effect by adrenaline on the uptake of fructose in the diaphragm was not as constant as the effect on the uptake of glucose and mannose. A definite inhibition by adrenaline was first obtained with a high concentration of fructose in the medium. The physiological importance of this adrenaline effect can therefore be somewhat uncertain. It should, however, be mentioned that BULLOUGH (1955)[1] has shown that adrenaline inhibits the mitosis in the epidermis in vitro with both glucose and fructose respectively in the medium.

The main problem in the present work has however been an investigation concerning the mechanism of the inhibition of the uptake of glucose in the diaphragm by adrenaline. It has been ascertained that this is not due to factors such as liberation of hexokinase from the diaphragm into the medium or the liberation of reducing substances. The examinations have also thrown some light on two possibilities for the adrenaline effect:

[1] Personal communication.

1) Is the effect secondary in relation to an accumulation of hexosemonophosphate?

2) Has adrenaline a primary effect on the hexokinase reaction?

The first possibility became feasible since it was demonstrated that glucose-6-phosphate was a strong inhibitor for animal hexokinase. WEIL-MALHERBE and BONE (1951), thus found approx. 50 per cent inhibition of dissolved brain hexokinase at 1×10^{-3} M G-6-P. For the brain hexokinase of particle structure CRANE and SOLS (1953), found 17 per cent inhibition at 2×10^{-4} M G-6-P and 50 per cent inhibition at 6×10^{-4} M G-6-P. JERVELL, WALAAS and WALAAS (1955), showed that 8×10^{-4} M G-6-P gave 50 per cent inhibition of the dissolved hexokinase from rat diaphragm at a concentration 1.2×10^{-3} M of ATP in the system. On increasing the concentration of ATP, the inhibition was at any rate partly released. Thus 1.6×10^{-3} M G-6-P gave an inhibition of 50 per cent at a concentration 2.3×10^{-3} M of ATP in the system.

In the usual aerobic incubation of the diaphragm, the content of hexosemonophosphate after completed incubation was 1.45×10^{-3} M normally, and 30 per cent higher after the addition of adrenaline, *i. e.* a range of concentration of G-6-P which gives a considerable inhibition of the dissolved hexokinase. SUTHERLAND (1952) also assumed that the intracellular concentration of hexosemonophosphate is a regulator in the uptake of glucose in muscle.

The effect of adrenaline on the diaphragm when the respiration is blocked by NaCN may contribute to an understanding of the problem. In this case the concentration of hexosemonophosphate in the diaphragm was reduced to $1—2 \times 10^{-4}$ M and any effect of adrenaline on the content could not be detected. The glucose uptake in the diaphragm from control animals under aerobic conditions and under the influence of NaCN was of the same magnitude. Further adrenaline gave an inhibition of the uptake of glucose not only in aerobic conditions, but also after the addition of NaCN. Both without and with addition of adrenaline more than 90 per cent of the uptake of glucose and degraded glycogen was recovered as lactic acid on the addition of NaCN to the medium.

According to these experiments it may be questioned if the intracellular concentration of hexosemonophosphate is of essential importance in the regulation of the uptake of glucose by the

isolated rat diaphragm. The considerably larger accumulation of hexosephosphates which have been observed in isolated frog muscles (HEGNAUER and CORI 1934) and in vivo in the muscle of rats injected with adrenaline (CORI, FISHER and CORI 1929) may on the other hand probably be of some importance in regulation of glucose assimilation.

It must be concluded that the inhibition of the uptake of glucose by adrenaline in the diaphragm may be at any rate partly due to a primary effect on the hexokinase reaction. The experiments which have been carried out on the effect of adrenaline on hexokinase from the diaphragm have so far only partly confirmed this hypothesis. The adrenaline did inhibit the hexokinase, but the effect was slight and not completely reproducible. In this connection it should be mentioned that RANDALL (1946) found that adrenaline inhibited the glycogenolysis in brain homogenate to a degree of 10 per cent. This corresponds to the inhibition reported in the present experiments. The reason for the slight adrenaline effect may be that hormonal effects on the cell metabolism are reproduced with difficulty in enzyme systems after disintegration of the tissue as is known from the effect of adrenaline on phosphorylase (SUTHERLAND and CORI 1951).

Summary.

1. The effect of adrenaline on the uptake of hexose in the isolated diaphragm, and the mechanism of the inhibition of the uptake of glucose by adrenaline have been investigated in the present work.

2. The uptake of hexose in the isolated diaphragm exhibits the following sequence glucose > mannose > fructose. Adrenaline inhibits the uptake of mannose to the same degree as glucose. With high concentrations of fructose in the medium adrenaline also causes an inhibition of the uptake of fructose.

3. The mechanism of the adrenaline effect is studied in experiments where the respiration is blocked by the addition of NaCN. The carbohydrate metabolism in the diaphragm then exhibits the following features.

a) The uptake of glucose in control experiments is of the same magnitude as under aerobic conditions. Adrenaline inhibits the uptake of glucose on an average by 25 per cent, an effect which

is approximately of the same magnitude as the adrenaline effect under aerobic conditions.

b) The concentration of hexosemonophosphate is rapidly reduced to 1/10 of the value under aerobic conditions and adrenaline has no effect. This is in contrast to the situation under aerobic conditions where adrenaline effects an accumulation of hexosemonophosphate.

4. Adrenaline has no definite effect on the content of inorganic P, ATP or phosphocreatine in the diaphragm.

5. Adrenaline produces a slight inhibition of the hexokinase activity in homogenate and extract of the diaphragm with an average value of 10 per cent.

6. The present work indicates that the inhibition of the glucose assimilation in muscle by adrenaline is not only due to an accumulation of hexosemonophosphate intracellularly, but can also be attributed to a primary effect of adrenaline on the hexokinase reaction.

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The Glycogenolytic Effect of Adrenaline on the Isolated Rat Diaphragm.

By

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The glycogenolytic effect of adrenaline in vitro on the isolated rat diaphragm has been described by RIESSER (1947) and by WALAAS and WALAAS (1950), thereby confirming a similar adrenaline effect on frog muscle in vitro described by HEGNAUER and CORI (1934).

Outstanding contributions to the understanding of the mechanism of the glycogenolytic effect of adrenaline, supported by investigations with isolated liver slices have been made by SUTHERLAND and CORI (1951). It was shown that adrenaline increased the concentration of active phosphorylase. This effect occurred with great velocity and could therefore be presumed to be of importance to the glycogen breakdown which takes place after the injection of adrenaline in vivo.

The present work has had the purpose of elucidating the glycogenolytic effect of adrenaline on the surviving muscle with an intact cell structure. This has been attained by the study of the effect on rat diaphragm under various experimental conditions.

Experimental.

Young male rats weighing 100—140 g have been used. The animals were killed after fasting for 24 hours and the diaphragm incubated in Krebs-Ringer-phosphate medium, pH 7.4. The experimental conditions are otherwise given in the tables.

The analyses of glycogen were carried out as described by WALAAS and WALAAS (1950). The method of NELSON (1944) was used for the analysis of glucose. Otherwise methods employed are described in detail in the previous paper (WALAAS 1955).

Results.

In experiments described in Fig. 1 the quantitative figures of the glycogenolytic effect of adrenaline are given. With 0.25 μg adrenaline per ml medium, half of the maximum effect was obtained while the maximum effect was reached with 1 μg per ml. In isolated liver slices, 0.05 μg of adrenaline produced half the maximum effect on the glycogenolysis (SUTHERLAND and CORI 1951). When a dose 5 times larger is required in experiments on the isolated diaphragm it can probably be ascribed to difference in penetration.

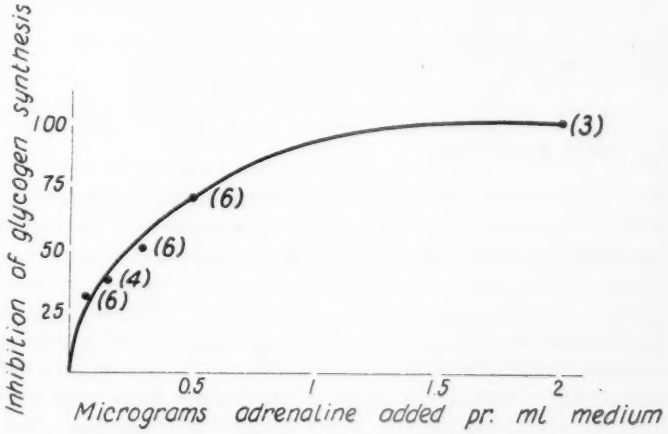

Fig. 1. The inhibition of the glycogen synthesis in rat diaphragm by adrenaline using different concentrations during the incubation. Incubation in 1 ml medium with 140 mg glucose per 100 ml at 37° C for 60 mins., 100 per cent O_2. Inhibition of the glycogen synthesis expressed in per cent of maximum inhibition. Maximum inhibition: 1.6 mg per g per hour = 100. The points represent mean values. Number of observations in ( ).

The glycogenolytic effect of adrenaline in the diaphragm after various times of incubation is shown in Fig. 2. Even after 5 mins. the breakdown of glycogen in the diaphragm was at a maximum. The glycogen content was kept unchanged by continued incuba-

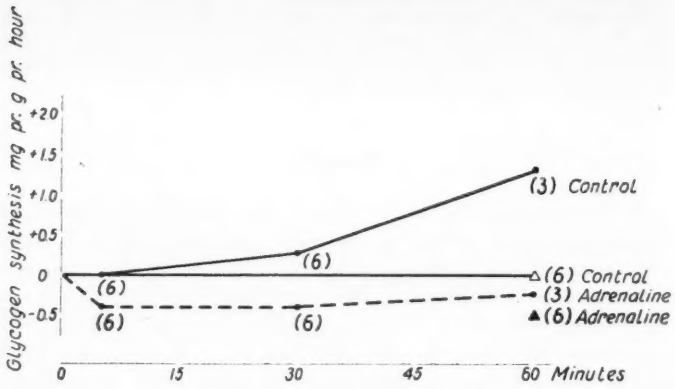

Fig. 2. Time relationship curve for the glycogenolytic effect of adrenaline on rat diaphragm.

Experimental conditions:

I) Incubation in 1 ml of medium containing 140 mg glucose per 100 ml for 60 mins. at 37° C, 100 per cent O_2.
2 micrograms adrenaline added.
The results stated: ——— · ——— = control,
- - - - - - - - - - - - - - - = adrenaline.

II) As given in I) but without the addition of glucose to the incubation medium. Only values after 60 mins. incubation are recorded. The results stated: △ = control, ▲ = adrenaline.

tion, with a slight rise when the incubation was undertaken for 60 mins. A continuous synthesis of glycogen occurred in the normal diaphragm upon increasing the time of incubation, and as previously shown, this stands in constant relation to the uptake of glucose (WALAAS and WALAAS 1950, KRAHL 1951). While the experiments mentioned were carried out in a glucose-containing medium, experiments in a medium free of glucose showed that the glycogen content in control experiments were unchanged after 60 mins. of incubation (Fig. 2) and that the glycogenolysis after the addition of adrenaline, was of the same magnitude as when glucose was present.

It appears from Fig. 3, that the glycogen synthesis in the normal diaphragm increased considerably on the increase of glucose concentration in the medium. When the glucose concentration in the medium was 500 mg per cent, there was also a slight glycogen synthesis after the addition of adrenaline. Thus, an increased glucose concentration in the medium partly reversed the glycogenolytic effect of adrenaline on the diaphragm. How-

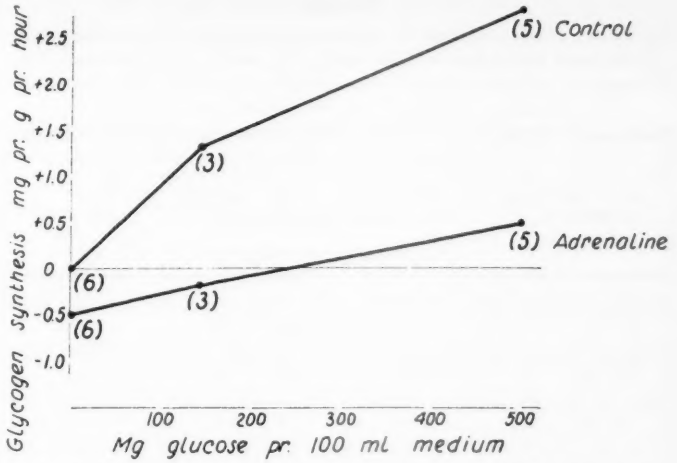

Fig. 3. The inhibition of the glycogen synthesis in rat diaphragm by adrenaline with various concentrations of glucose in the medium. Incubation in 1 ml of medium for 60 mins. at 37° C, 100 per cent O_2. 2 micrograms adrenaline added in vitro.

ever, the difference between the final glycogen content in the diaphragm, was greater with high than with low glucose concentration in the medium.

The glycogenolytic effect of adrenaline was also examined when mannose or fructose were used as substrate during the incubation (Table 1). In both cases, there was a definite inhibition of the glycogen synthesis. With low fructose concentration in the medium there was glycogenolysis, and with high concentration of fructose a slight glycogen synthesis. In the experiments with mannose as substrate a slight glycogen synthesis took place after addition of adrenaline. Principally there appears to be no difference in the adrenaline effect when the three hexoses, glucose, mannose and fructose are used as substrate.

Under a series of different experimental conditions the glycogenolytic effect of adrenaline on the diaphragm was further examined. The importance of phosphate in the incubation medium is made clear in experiments where bicarbonate buffer has been used without the addition of phosphate (Table 2). The adrenaline effect in this case was as in phosphate buffer (Table 3, group II). Thus a maximum glycogenolytic effect in the diaphragm is ob-

Table 1.

The effect of adrenaline on the formation of glycogen in rat diaphragm with mannose and fructose in the incubation medium. The concentrations of mannose and fructose in the incubation medium have been mentioned. Incubation 60 mins. at 37° C, 100 per cent O_2.

Substrate in the medium	Glycogen formation			
	Without Adrenaline	With Adrenaline	Difference	Statistical test
	mg per g per hour			
Mannose 140 mg per 100 ml	1.4 ± 0.08[1] (6)	0.15 ± 0.13 (6)	— 1.25	t: 8, P<0.001
Fructose 140 mg per 100 ml	0.4 ± 0.09 (10)	— 0.2 ± 0.15 (10)	— 0.6	t: 3.4, P: 0.003
Fructose 500 mg per 100 ml	1.8 ± 0.19 (9)	0.5 ± 0.20 (9)	— 1.3	t: 4.6, P<0.001

2 micrograms adrenaline added.
Number of observations in ( ).
[1] Standard error of mean.

Table 2.

The effect of adrenaline on the formation of glycogen in rat diaphragm incubated in a medium containing bicarbonate buffer. Incubation in 1 ml Krebs-Ringer-bicarbonate medium, pH 7.4 (without addition of phosphate), 140 mg glucose per 100 ml. Incubation 60 mins. at 37° C, 100 per cent oxygen. 2 micrograms adrenaline added.

Glycogen formation			
Without Adrenaline	With Adrenaline	Difference	Statistical test
mg per g per hour			
1.2 ± 0.07 (6)	— 0.4 ± 0.15 (6)	— 1.6	t: 9.4 P < 0.001

tained without the addition of phosphate, as distinct from conditions in isolated liver slices (SUTHERLAND 1950).

The normal glycogen synthesis in the diaphragm is reduced by an increase in the concentration of potassium in the medium (Table 3) since the glucose uptake was simultaneously reduced (E. WALAAS 1955). However adrenaline produced no increased breakdown of glycogen in the diaphragm when the concentration

Table 3.

The effect of adrenaline on the formation of glycogen in the diaphragm with various concentrations of sodium and potassium in the medium. Incubation in 1 ml medium containing different amounts of Na^+ and K^+ (the sum was always constant = 152 m. eq. per l).

Group	Incubation medium Per cent of: Na^+	K^+	Glycogen formation: Without Adrenaline	With Adrenaline	Difference	Statistical test
			mg per g per hour			
I	100	0	1.7 ± 0.11 (8)	0.5 ± 0.10 (8)	— 1.2	t: 8, P<0.001
II	95	5	1.45 ± 0.16 (11)	— 0.25 ± 0.07 (10)	— 1.7	t: 9.4, P<0.001
III	50	50	0.6 ± 0.13 (5)	— 0.1 ± 0.13 (5)	— 0.7	t: 3.8, P: 0.005
IV	0	100	0.2 ± 0.15 (7)	0 ± 0.13 (7)	— 0.2	t: 1.0, P: 0.3

The medium contained Mg^{++} in usual concentrations (1.5 m. eq. per l), phosphate buffer pH: 7.4, 140 mg glucose per 100 ml. Incubation 60 mins. at 37° C, 100 per cent oxygen, 2 micrograms adrenaline added.

of potassium was increased. Thus the experiments give no certain clues as to whether the effect of adrenaline is determined by the presence of potassium in the medium.

The effect of adrenaline on the diaphragm has also been examined when n-butanol was added to the medium (Table 4). In control experiments the glycogen synthesis was reduced to half of the normal synthesis and the glycogenolytic effect of adrenaline was increased. Thus adrenaline gave a breakdown of glycogen of 50 per cent of the initial content in these experiments as compared to a maximum glycogenolytic effect of 25 per cent without n-butanol. The increased glycogenolytic effect of adrenaline after the addition of n-butanol could not be explained by any influence on the glucose uptake. It appears from Table 4 that the uptake of glucose in control experiments as well as after the addition of adrenaline was of the same magnitude with and without addition of n-butanol to the medium.

Experiments where the cell structure had been destroyed by preliminary freezing and thawing of the diaphragm (Table 5) were carried out. A liberation of small amounts of reducing substances occurred into the medium during the incubation, and

Table 4.

The effect of adrenaline on the formation of glycogen in rat diaphragm when the medium contains n-butanol. Incubation in 1 ml medium with 140 mg glucose per 100 ml. The medium contained 1 ml n-butanol per 100 ml. Incubation 60 mins. at 37° C, 100 per cent oxygen. 2 micrograms adrenaline added.

Glucose uptake[1]			Glycogen formation			
Without Adrenaline	With Adrenaline	Difference	Without Adrenaline	With Adrenaline	Difference	Statistical test
mg per g per hour			mg per g per hour			
2.15±0.20 (9)	1.6±0.27 (9)	−0.55	0.7±0.19 (10)	−0.9±0.10 (10)	−1.6	t: 7.5, $P < 0.001$

[1] Uptake of glucose in the diaphragm without the addition of n-butanol is in controls 2.5 ± 0.18 and after adrenaline 1.9 ± 0.12 (E. WALAAS, 1955).

Table 5.

Liberation of reducing substances and the breakdown of glycogen in rat diaphragm after freezing and thawing. The diaphragm was frozen at − 70° C between two blocks of solid carbon dioxide for 5 mins. and thawed in the medium at 37° C. Incubation in 1 ml medium with 140 mg glucose per 100 ml for 60 mins. at 37° C, 100 per cent oxygen. 2 micrograms adrenaline or 0.1 unit insulin added.

Reducing substances formed[1]			Glycogen breakdown			Final glycogen content
Control	With Adrenaline	With Insulin	Control	With Adrenaline	With Insulin	Control
mg per g per hour			mg per g per hour			mg per g
0.5 ± 0.18 (6)	0.6 (3)	0.3 (3)	0.3 ± 0.06 (6)	0.3 (3)	0.3 (3)	0.15 ± 0.04 (6)

[1] Expressed in glucose equivalents.

after the experiment the glycogen had almost completely disappeared. The addition of adrenaline or insulin had no definite effect upon the carbohydrate metabolism under the experimental conditions employed.

An antagonistic effect of adrenaline and insulin upon the formation of glycogen in the diaphragm has also been demonstrated (Table 6). By pre-exposure of the diaphragm with adren-

Table 6.

The antagonistic effect of adrenaline and insulin on the glycogen synthesis in the isolated diaphragm. Incubation in 1 ml medium containing 140 mg glucose per 100 ml for 60 mins. at 37° C, 100 per cent O_2.

Group I		Group II		Group III	
Without Adrenaline	With Adrenaline	With Adrenaline	With Adrenaline + Insulin	With Insulin	With Insulin + Adrenaline
Glycogen synthesis, mg per g per hour					
1.7 (3)	0.9 (3)	0.7 ± 0.23 (6)	1.3 ± 0.25 (6)	4.7 ± 0.32 (6)	2.9 ± 0.19 (6)

Group I: The diaphragm had been pre-exposed to a medium without and with 2.7×10^{-4} M of adrenaline for 5 mins. at 25° C. Washed twice with 25 ml of incubation medium. At the final incubation of the diaphragm adrenaline was not added.

Group II: The diaphragm had been pre-exposed to a medium without and with 5.5×10^{-4} M of adrenaline for 5 mins. at 25° C. Washed twice with 25 ml of medium. The final incubation was carried out without and with the addition of insulin, one unit per ml, respectively. Statistical test: t: 1.8, P: 0.10.

Group III: The diaphragm had been pre-exposed to a medium containing one unit of insulin per ml for 5 mins. at 25° C. Washed twice with 25 ml of medium. The final incubation was carried out without and with the addition of adrenaline, 2 micrograms per ml, respectively. Statistical test, t: 4.7, P: 0.001.

aline, an inhibition of the glycogen synthesis was found, but the effect was only 50 per cent of the effect when adrenaline had been added during the incubation and a higher dose, 50 μg per ml had to be used. This effect by pre-exposure with adrenaline was almost cancelled on the addition of insulin at the final incubation. Simultaneously the uptake of glucose was increased. It was also demonstrated that the stimulation of the glycogen synthesis by pre-exposure to insulin was inhibited on the addition of adrenaline during the final incubation.

Discussion.

The investigations indicate that the glycogenolytic effect of adrenaline is a primary one on the muscle metabolism. The effect is independent of the supply of substrate since it is present at the incubation of the diaphragm in a glucose-free medium. The effect has no particular relationship to the inhibiting effect upon the glucose uptake (Walaas and Walaas 1950) since adrenaline

even after a few minutes' incubation results in breakdown of glycogen in the diaphragm. Previous investigations on muscle (HEGNAUER and CORI 1934, SUTHERLAND 1952) and isolated liver slices (SUTHERLAND and CORI 1951) have likewise demonstrated that the glycogenolytic effect of adrenaline is a primary effect on the metabolism.

The investigations carried out by SUTHERLAND and CORI on liver, have shown that an increase in the amount of active phosphorylase is a factor of importance for the glycogenolytic effect by adrenaline. Further investigations by SUTHERLAND (1952) have shown that in rat diaphragm, adrenaline inhibits the transformation of phosphorylase a $\longrightarrow$ b or that this transformation is reversed. The reason for the fact that adrenaline in vivo always gives a breakdown of glycogen cannot however be immediately explained by the aforementioned facts.

The present experiments have demonstrated that the glycogenolytic effect of adrenaline in the diaphragm is particularly characterized by two facts:

Firstly, the effect takes place very rapidly since the breakdown of glycogen is already at a maximum after a few minutes of incubation. Secondly, the glycogen breakdown is only partial and does not exceed 20—30 per cent of the initial glycogen content. In this connection it may be of interest to mention that the initial phosphorylytic degradation of the glycogen to the "limit dextrin stage" composes approximately 30 per cent of the glycogen molecule (CORI and LARNER 1951). It should also be pointed out that when the degradation of glycogen approaches this stage a change occurs in the equilibrium of the phosphorylase reaction to the side of polysaccharide formation (CORI and CORI 1947, HESTRIN 1949).

In contrast to the breakdown of glycogen produced by adrenaline there occurs an almost complete degradation of glycogen in the isolated diaphragm when incubated under anaerobic conditions (WALAAS and WALAAS 1952) and after the addition of 2,4-dinitrophenol (JERVELL, WALAAS and WALAAS 1955). This almost complete glycogenolysis is obviously related to the considerable increase in the concentration of inorganic phosphate which simultaneously occurs in these experiments and reverses the phosphorylase reaction until the glycogen depot is exhausted. On the other hand no increase in the concentration of inorganic phosphate in the diaphragm could be detected after the addition

of adrenaline (WALAAS 1955), and this fact may be of importance when the breakdown of glycogen in this case is only partial.

Otherwise it is characteristic that adrenaline effects an accumulation of hexose monophosphate in the diaphragm when incubated under aerobic conditions (SUTHERLAND 1952, E. WALAAS 1955). This effect of adrenaline was originally demonstrated in vivo in rat muscle after the injection of adrenaline (CORI and CORI 1931) and later in frog muscle in vitro (HEGNAUER and CORI 1934). Thus it can be concluded that adrenaline influences the reactions in the diaphragm in vitro in a manner which is characterized by the following: In the course of a few minutes 25 per cent of the initial glycogen content in the diaphragm is broken down. On continued incubation a new metabolic state of equilibrium appears, characterised by a low and almost constant content of glycogen and an increased content of hexose monophosphate.

When the glucose concentration was increased in the medium during the incubation the adrenaline effect was partly reversed. A slight glycogen synthesis was recorded under these conditions in experiments where adrenaline was added. When it concerns the mechanism of the effect of adrenaline, SUTHERLAND has stressed the importance of activation of the phosphorylase. Experiments designed to detect an increase in the concentration of inorganic phosphate after the addition of adrenaline have failed. On the contrary the content is reduced in frog muscle (HEGNAUER and CORI 1934) while it was unchanged in the diaphragm. These determinations however do not permit the drawing of any conclusion concerning the amount of inorganic phosphate which takes part intracellularly in the phosphorylase reaction. The slight reduction of creatine phosphate detected in the diaphragm (WALAAS 1955) does not permit any conclusions to be drawn either. The problem of why adrenaline always accelerates the metabolism of glycogen in the direction of a breakdown thus still remains open.

LESSER (1923) put forward the theory that the effect of adrenaline on the metabolism was due to stimulation of enzyme reactions by the liberation of enzymes bound in the cell, an explanation which was assumed for the metabolic effects of alcohols. SUTHERLAND (1950) found however, no increased glycogenolysis and no increased glycogenolytic effect of adrenaline on isolated liver slices treated with alcohols or detergents. On

the other hand the present investigations are more in agreement with LESSER since n-butanol inhibited the glycogen synthesis in the diaphragm and increased the glycogenolytic effect of adrenaline. The changes which are responsible for this effect of n-butanol are not known. By advanced destruction of the cell structure in the diaphragm by freezing and thawing, the ability to synthesise glycogen was completely lost.

Summary.

1. The glycogenolytic effect of adrenaline on the isolated rat diaphragm is characterized by:

a) The breakdown of glycogen is partial and does not exceed 25 per cent of the initial glycogen content.

b) The glycogenolysis occurs rapidly and is at a maximum after 5 mins.

c) The degree of glycogen breakdown is approximately the same in glucose-free or glucose-containing medium (140 mg per cent) while the increase in glucose concentration in the medium (500 mg per cent) partly counteracts the effect of adrenaline.

d) The effect also occurs with mannose or fructose as substrate during incubation.

e) The effect is at a maximum without requiring the addition of inorganic phosphate to the medium.

2. The increase of the potassium concentration in the incubation medium inhibits the uptake of glucose and the glycogen synthesis in the diaphragm. The breakdown of glycogen in the diaphragm after the addition of adrenaline is not more pronounced with a high potassium concentration in the medium.

3. n-Butanol affects the inhibition of the normal glycogen synthesis, and the glycogenolytic effect of adrenaline becomes also more pronounced. After freezing and thawing of the diaphragm the ability of glycogen synthesis is abolished.

4. On addition of adrenaline in vitro, a new metabolic equilibrium in the diaphragm takes place, characterized by a lower glycogen content and an increased content of hexose monophosphate. The mechanism of the adrenaline effect is discussed.

Acknowledgement is made to Nordisk Insulinfond for a grant.

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Fibrinolytic Activity in Human Saliva.

By

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Several investigations during the last years have shown the fibrinolytic system in the organism to be very complicated (for references see ASTRUP, 1954). The current view of the system in the blood (MÜLLERTZ and LASSEN, 1953) is presented in the following scheme:

The fibrinolytic enzyme, plasmin, normally occurs in the blood as a precursor, plasminogen. The transformation from plasminogen to plasmin can be caused by a number of activators, which are especially found in certain tissues ("fibrinokinase"). Furthermore, there exists in normal blood a precursor of a plasminogen activator. The transformation from precursor (proactivator) to activator is effected by a lysokinase. Lysokinases are present in certain tissues. Streptokinase acts as a lysokinase. Inhibitory compounds against this fibrinolytic system occur normally in the blood.

Recent investigations have demonstrated the presence of an activating fibrinolytic system in human urine (WILLIAMS 1951, ASTRUP and STERNDORFF 1952, SOBEL et al. 1952), milk (ASTRUP

and STERNDORFF 1953), and lacrymal fluid (STORM 1955). This system resembles the system in the blood but differs in that normally no or only very small amounts of plasminogen and inhibitory agents are present.

The present paper reports a similar system in human parotid saliva and in mixed saliva from the mouth.

Materials and Methods.

Saliva from the parotid gland was collected in small plastic cups as described by CURBY (1953). By this method it is possible to collect parotid saliva not contaminated with secretions from other oral glands. Mixed saliva was collected directly from the mouth.

The standard fibrin plate method of ASTRUP and MÜLLERTZ (1952) was used for the examination of the saliva. The bovine fibrin in these plates contains large amounts of plasminogen which reacts with plasminogen activators. For this reason the activities recorded on the standard fibrin plates gives a measure of the combined activities of plasminogen activator and plasmin in a sample. When the plasminogen in the standard fibrin plates is destroyed by heating to 85° for 35 minutes, plasmin may be determined alone (LASSEN 1952). The presence of proactivator may be tested by the addition of streptokinase, which transforms proactivator into activator (MÜLLERTZ and LASSEN, 1953) in the same way as the lysokinases of tissue. The activity was recorded as the diameter product (in mm^2) of the lysed zone after 20 hours digestion at 37° (average of 3 determinations).

Streptokinase ("Varidase") was obtained from the Lederle Laboratories. The stock solution contained 1 mg/ml diluted 1 to 25 in barbital buffer (pH 7.8).

Bovine plasminogen was prepared as described by ASTRUP and STERNDORFF (1953). The stock solution contained 20 mg/ml in barbital buffer (pH 7.8).

Pure crystalline trypsin was obtained from the Novo Laboratories, Copenhagen. The stock solution contained 0.25 mg/ml diluted 2 to 15 in barbital buffer (pH 7.8).

Results and Discussion.

The production of saliva from the parotid gland was stimulated by sucking boiled sweets and the parotid secretion was tested immediately after collection. Salivary flow during stimulation ranged from 0.5 to 2.0 ml per minute.

When samples (from 10 different persons) were applied to standard fibrin plates (containing plasminogen) no or only very slight activities were recorded. Addition of diluted streptokinase solu-

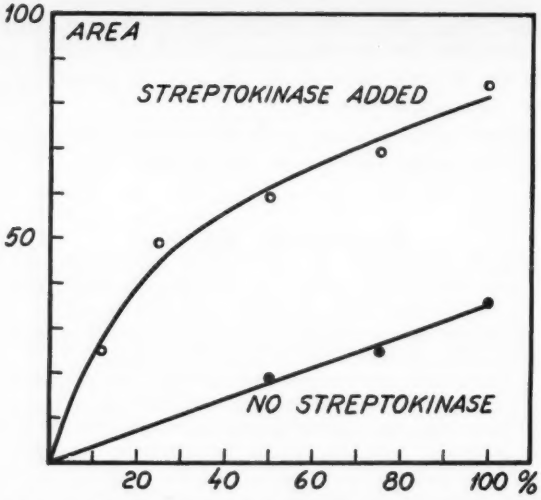

Fig. 1. Activity of serial dilutions of parotid saliva on standard fibrin plates before and after addition of streptokinase. *Abscissa:* Concentration of saliva in % of stock solution. *Ordinate:* Activity as product of 2 diameters (mm^2) of the lysed zones.

tion (0.5 vol.) to the samples produced an increase in the activity on the standard fibrin plates, while streptokinase alone had no effect (fig. 1). No activity was recorded when samples were applied to heated fibrin plates. These results demonstrate the absence of fibrinolytic enzyme, the presence of varying and very small amounts of a plasminogen activator and of a considerable amount of a precursor to a plasminogen activator.

Addition of streptokinase solution (0.5 vol.) to the samples produced no activity on heated fibrin plates. Addition of bovine plasminogen (0.5 vol.) produced lysis and this activity was further increased by addition of streptokinase (0.5 vol. containing plasminogen 20 mg/ml) (fig. 2). These results indicate the absence of plasminogen in the samples and confirm the above results that small amounts of a plasminogen activator and large amounts of a precursor of a plasminogen activator were present.

The samples of parotid saliva from the same person before and during stimulation were compared. The flow from the unstimulated parotid gland was 0.03—0.10 ml/min. The activities in the unstimulated samples (7 different persons) were slightly

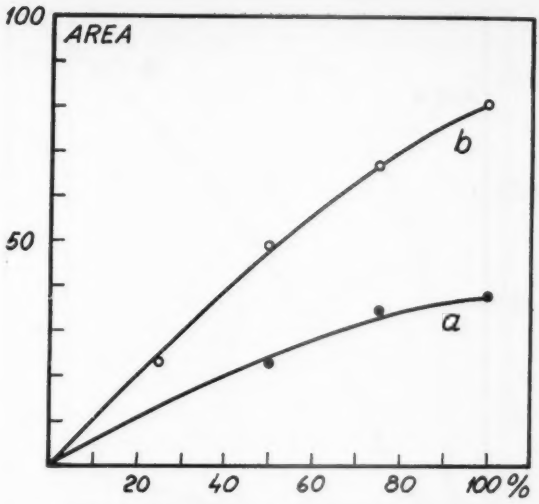

Fig. 2. Activity of serial dilutions of parotid saliva on heated fibrin plates.
Parotid saliva alone: no activity.
Parotid saliva + bovine plasminogen (a).
Parotid saliva + bovine plasminogen + streptokinase (b).
Abscissa and ordinate: like fig. 1.

higher than in the samples collected during stimulation, and addition of streptokinase solution produced a larger increase in the activity obtained on the standard fibrin plates in the unstimulated samples than in the stimulated parotid saliva (fig. 3).

When samples of mixed saliva from the mouth (6 different persons) were applied to the fibrin plates similar results were recorded.

In a search for possible trypsin inhibitors all samples of stimulated parotid saliva, unstimulated parotid saliva and mixed saliva from the mouth were added to a solution of trypsin (1.0 vol.) and applied to heated fibrin plates. We were only able to record a slight trace of inhibitory activity against trypsin in one single sample of unstimulated parotid saliva. All other samples showed no inhibitory activity.

In order to characterize the plasminogen activator formed by addition of streptokinase we have studied its stability at different temperatures and pH values. Streptokinase solution was added to samples of mixed saliva from the mouth and left for one hour

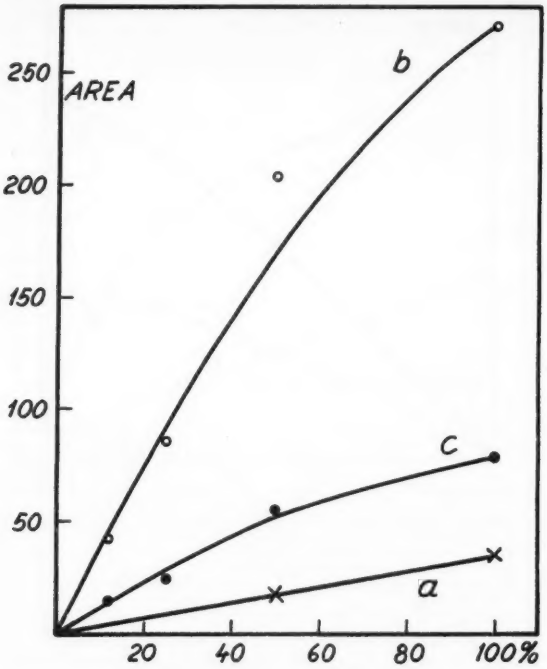

Fig. 3. Activity of serial dilutions of parotid saliva on standard fibrin plates.
Parotid saliva (unstimulated) (a).
Unstimulated parotid saliva + streptokinase (b).
Stimulated parotid saliva: no activity.
Stimulated parotid saliva + streptokinase (c).
Abscissa and ordinate: like fig. 1.

at room temperature. After regulating the pH value (using 0.1—N HCl, 0.1—N NaOH and different buffers) and heating for 30 min. at 37° C., 50° C., 70° C. and 100° C. the samples were cooled to 0° in ice water, neutralized and immediately applied to standard fibrin plates (after correcting the volumes) in order to estimate the remaining activity (fig. 4). The activator was partly destroyed at 50° C. and completely destroyed at 70° C. and 100° C. The optimal stability was found at pH 7.6. The activator was rather labile outside this pH value even at 37°. These results correspond with those obtained in lacrymal fluid and urine.

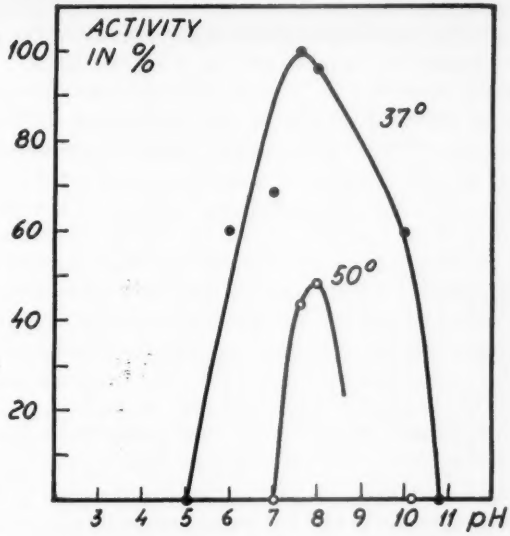

Fig. 4. Fibrinolytic activity of saliva on standard fibrin plates after addition of streptokinase and heating for 30 min. at 37° C. and 50° C.
Abscissa: pH values of the samples during heating.
Ordinate: Activity in % of unheated control.

The results demonstrate the existence of a fibrinolytic system in human parotid and mixed saliva similar to that found in milk, urine and lacrymal fluid. This system differs from the fibrinolytic system in the blood as large amounts of plasminogen and inhibitory compounds against trypsin are present in the blood. The present investigation does, however, not give any information as to the source of the salivary activator and proactivator. Two possibilities may be considered:

1. The substances are excreted by diffusion from the blood plasma to the lumen of the gland. In this case the glandular epithelium should be selectively permeable to the components of the fibrinolytic system of the plasma withholding plasminogen and trypsin inhibitor, but allowing activator and proactivator to penetrate into the saliva. The molecular sizes and charges of the different substances are not known, and it is therefore impossible to relate the present findings to current concepts of the permeability of the parotid epithelium. The difference between the activity in stimulated and in unstimulated parotid saliva

seems to support this conclusion because the same activity in the two samples would be expected if the activity in the parotid saliva was caused by simple filtration from the blood.

2. Another possibility is that the salivary activator and proactivator is produced in the parotid epithelium in the same manner as ptyalin and other specific salivary components. The decrease in the concentration of proactivator and activator during stimulation could then be caused by depletion of the glandular epithelium such as has been shown by BABKIN (1950) to occur with the enzymes in saliva and other secretions. Further work is needed in order to decide between these two possibilities.

A fibrinolytic system has been recorded in seminal fluid (KAULLA and SHETTLES 1953, LUNDQUIST, THORSTEINSSON and BUUS 1955), but further investigations are needed before a comparison with blood and the secretions mentioned can be made.

It is probable that the existence of this system in parotid saliva has a definite physiological significance. Like human urine, lacrymal fluid and milk, parotid saliva has to pass through a narrow excretory duct, and the formation of fibrin clots in this duct would produce stagnation of secretion. KUSSMAUL (1879) has reported such a case of obstruction of the duct from the parotid gland (sialodochitis fibrinosa). The plasminogen activator present in parotid saliva can therefore be of aid in the resolution of eventually formed fibrin clots in Ductus Parotideus Stenonii.

Summary.

1. Human parotid saliva and mixed saliva from the mouth have been investigated in a search for a fibrinolytic system similar to the systems found in human blood, milk, lacrymal fluid, urine and seminal fluid.

2. Small and variable amounts of a plasminogen activator and large amounts of a proactivator of plasminogen were found. Plasminogen was absent. A slight trace of inhibitory activity against trypsin was recorded in only one sample. All other normal samples showed no inhibitory activity.

3. The fibrinolytic system in saliva thus differs from the system in blood and is similar to those in other secretions.

4. The activity was larger in unstimulated parotid saliva than in the stimulated parotid saliva from the same person.

Acknowledgement.

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The Binding of Histamine to Phosphatides.

By

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It is a well established fact that many tissues and organs contain histamine. It may be that this bound histamine is rendered pharmacologically inactive by the direct attachment to a cell or plasma component. This is implied by many experimental findings. Thus ROCHA e SILVA (1942) has suggested that histamine combines, by a simple chemical linkage, with free carboxyl groups in protein molecules. According to PARROT (1953) certain components of serum, especially γ-globulin, are capable of binding histamine in vitro. However, KAPLAN (1953) was not able to verify these findings using solutions of human γ-globulin, β-globulin and plasma albumin as well as normal human serum.

As to the localization in the cells it has been suggested that bound histamine is restricted to the mitochondria (COPENHAVER 1953, HAGEN 1954).

In an earlier investigation (LINDAHL 1955), it was shown *in vitro* that histamine enters into combination with certain phosphatides to form an ether soluble complex. As phosphatides appear in most tissues, *e. g.* as the prostetic group of lipoproteins, this complex formation might be of some general interest. Therefore, it seemed worth while to reinvestigate the question. In this paper the conditions under which the binding of histamine takes place are described, as well as the influence of some different factors.

Materials and Methods.

Preparation of *lecithin* was accomplished according to PANGBORN (1951), which preparation is considered to be the best method in use for preparing large quantities of pure lecithin. On paper chromatography it still showed minute amounts of ninhydrin-reactive material.

Cephalin was prepared from calf brain using the method of FOLCH (1942). The product is said to contain phosphatidylserine, phosphatidyletanolamine, and diphosphoinositide.

Histamine stock solutions, containing 1 mg of free base per ml in 0.01-M HCl, were prepared from histamine diphosphate (BDH) or from the hydrochloride (Usines Chim. Lab. Français). All amounts of histamine are expressed in terms of free base. For the composition of the *buffer solutions* see BAMANN-MYRBÄCK (1941).

Assay. Histamine was assayed on a piece of guinea pig ileum suspended in a 2 ml bath with Tyrode's solution to which atropine sulphate was added in the concentration $2.5 \cdot 10^{-7} : 1$.

Experimental procedure. Experiments were carried out with ether solutions or with water suspensions of the phosphatides.

Procedure I.

Samples of phosphatide were dissolved in ether and mixed by gentle shaking with the same volume of an aqueous solution of histamine in buffer. When the mixture had separated in two phases, half of the water phase was drawn off from the bottom and neutralized when wanted. The small amounts of dissolved ether were removed by passing a current of air through the sample. The amount of histamine remaining in the water phase was then assayed. The ether phase was shaken with a salt solution of the same composition as a Tyrode, with the exception that the concentrations were doubled and no $NaHCO_3$ was used. The water phase was drawn off, restored to the proper salt concentration and treated as above.

In all experiments controls were worked parallel to the samples: the buffer solution without histamine was treated with the ether-phosphatide solution and a sample of histamine buffer was treated with ether. After separation of the two phases in both controls, the water resp. ether phases were mixed together, and treated in the same way as the samples.

Procedure II.

The phosphatides were suspended in buffer and added to histamine, dissolved in the same buffer. After shaking, the mixture was centrifuged at 13,000 g and the clear supernatant was analysed for histamine after proper neutralising and diluting. Controls were carried out on the same principle as before.

Results.

In table I are presented the results of some experiments carried out with phosphatide dissolved in ether.

Table I.

Histamine conc. 2 µg/ml in 0.024-M $NaHCO_3$.

Buffer	Phosphatide concentration in ether g/100 ml	Histamine left in buffer after treatment %	Histamine recovered from ether %	Total recovery %
Lecithin	8	61	30	90
Controls	8	100	0	100
Cephalin	0.9	60	21	81
Controls	0.9	100	0	100

The results represent the mean value for 5 different experiments. The individual variations do not exceed the experimental error for the bioassay.

Variation in pH.

Preliminary experiments had shown that different buffers at the same pH give essentially the same results, provided the ionic strength is about the same. For this reason, some of these experiments could be performed by using different buffers in order to obtain the wanted pH range. Histamine and phosphatide-concentrations were kept constant.

In table II the results of the experiments with the phosphatide in ether solution are given.

Table II a.

Histamine conc. 1 µg/ml, lecithin conc. 45 mg/ml.

Buffer	Carbonate 0.05-M bicarbonate 0.1-M	Prim. phosphate 0.05-M NaOH 0.2-M				Citrate 0.1-M-NaOH 0.1-M	
pH	9.7	8.0	7.8	7.5	6.9	5.9	4.0
Histamine left after treatment %	12	25	40	40	50	90	85

Table II b.

Histamine conc. 3 μg/ml, cephalin conc. 10 mg/ml Borax 0.05-M-NaH_2PO_4 0.05-M-buffer.

pH	9.0	8.4	8.0	7.4	7.0	6.4	5.8
Histamine left after treatment %	35	37	40	58	65	70	80
Histamine recovered from ether %	60	62	41	21	14	8	9

In table III the influence of pH on the binding of histamine to a phosphatide in suspension is shown.

Table III a.

Lecithin conc. 5 %; histamine conc. 10 μg/ml.

Buffer	Na_2CO_3 0.025-M $NaHCO_3$ 0.05-M		$Na_2B_4O_7$ 0.0125-M NaH_2PO_4 0.025-M			Na_2HPO_4 0.025-M NaH_2PO_4 0.025-M
pH	11.4	10.1	8.8	8.4	8.0	7.0
% histamine left after treatment ..	60	70	75	80	95	100

Table III b.

Cephalin conc. 4 %; histamine 10 μg/ml.

Buffer	Na_2CO_3 0.025-M $NaHCO_3$ 0.05-M			Na_2HPO_4 0.025-M NaH_2PO_4 0.025-M			
pH	11.2	10.0	8.9	8.1	7.2	6.5	4.5
% histamine left ...	5	10	15	20	26	40	33

Varied Concentrations.

Experiments were carried out with various histamine and phosphatide concentrations in buffer, pH and salt concentration being kept constant.

In table IV the amount of histamine bound per unit phosphatide at different concentrations is shown.

Table IV.

Buffer 0.025-M Na_2CO_3 pH 11.2.

	Phosphatid concentration mg/ml	Histamine concentration µg/ml	µg histamine bound per mg phosphatide
Cephalin	2	10	3
	2	5	1.6
	2	0.2	0.08
	40	10	0.2
	0.2	10	10
	0.02	10	50
Lecithin	40	10	0.08
	40	2	0.02
	40	0.4	0.006

In fig. 1 the correlation between bound histamine and histamine concentration in the solution is shown, the phosphatide being suspended in a buffer solution.

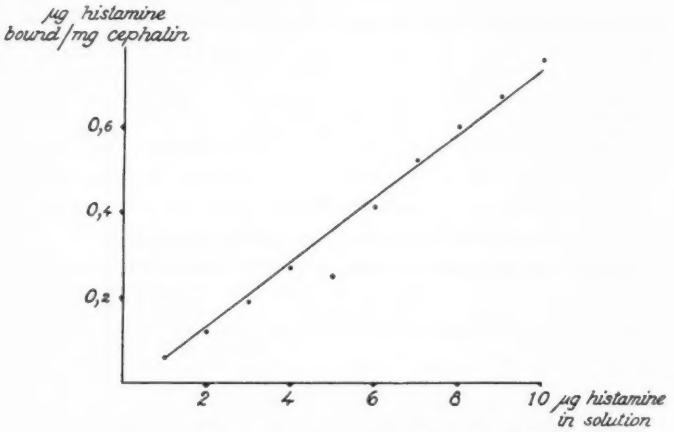

Fig. 1. Cephalin conc. 0.5 %, buffer 0.024 M $NaHCO_3$.

Varied Salt Concentration.

The binding of histamine by phosphatides in suspension at various concentrations of NaCl was determined at pH 11.2, the histamine and phosphatide concentrations being constant. The results are shown in table V.

Table V.

Na_2CO_3-buffer pH 11.2; histamine 10 µg/ml.

	NaCl conc. %	Histamine left %
Lecithin 4 %	2	70
	1	80
	0.5	75
	0	70
Cephalin 4 %	2	27
	1	21
	0.5	16
	0	10

Comments.

The experimental results obtained with procedure I indicate that histamine forms some complex with the lecithin and cephalin and thereby becomes ether soluble. The pH of the water phase has a great influence on the binding which suggests that the free phosphoric-acid group of phosphatidylic acid plays an important rôle in the formation of the histamine-phosphatide complex.

The experiments performed according to procedure II demonstrate that histamine is bound to the solid phase in a phosphatide suspension. With increasing pH the amount of histamine attached to the lecithin or the cephalin rises rapidly.

Sodium chloride does not seem to influence the binding of histamine by lecithin but has a definite effect in the case of cephalin. This is consistent with the findings of CHRISTENSEN (1940), who showed that cephalins in contrast to lecithins combine with sodium.

The amount of histamine which can be bound to a unit of phosphatide appears to depend both on the histamine and the phosphatide concentration. With increasing concentrations the binding capacity augments, as is to be expected in every reversible formation of a complex. The curve in fig. 1 is very nearly a straight line, which suggests that the complex formation attains an equilibrium of the type $A + B \rightleftharpoons AB$. However, the investigated concentrations are relatively low on account of the limits of the biological assay. Under these circumstances the application of an adsorption equilibrium equation might also give a straight line. A close study with different methods of a wide range of concentrations is needed to allow any statement to be made on this question. Consequently

the experiments presented so far do not make it possible to decide whether the histamine phosphatide complex is a true molecular compound or an adsorption complex.

Summary.

When an aqueous solution of histamine is treated with lecithin or cephalin in ether solution, part of the histamine disappears from the water phase but can be recovered from the ether phase. pH influences the distribution of the histamine between the ether and the water phase.

When lecithin or cephalin, suspended in buffer and added to a histamine solution, are removed by centrifugation part of the histamine adheres to the solid phase. The distribution of the histamine is influenced by pH and certain salts.

The experiments carried out so far do not make it possible to decide whether the phosphatide-histamine complex is a true molecular compound or and adsorption complex.

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On the Functional Organization of the two Presynaptic Systems to the Colonic Nerve Neurons of the Inferior Mesenteric Ganglion in the Cat.

By

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LLOYD (1937) showed that the colonic nerve from the inferior mesenteric ganglion of the cat is composed exclusively or almost exclusively of postganglionic fibers with their trophic centers in the same ganglion. The postganglionic fibers discharged after a volley in the inferior splanchnic nerves but LLOYD never found any discharge in the colonic nerve after stimulation of the hypogastric nerve. However, JOB and LUNDBERG (1952) demonstrated that an impulse could be elicited in the colonic nerve by stimulation of the hypogastric nerve. This impulse is synaptically relayed in the ganglion and the presynaptic fibers are small C-fibers, which have their trophic centers in the periphery. At least most of the fibers end in the ganglion (JOB and LUNDBERG, 1952). In the same year BROWN and PASCOE independently made similar observations on the rabbit.

After stimulation of the inferior splanchnic nerves the peripheral reflex elicited from the hypogastric nerve is strongly depressed for several hundred milliseconds. This was interpreted by JOB and LUNDBERG as due to a convergence of the two presynaptic systems on the same postsynaptic neurons and thus to a high degree of occlusion. BROWN and PASCOE, however, explained similar findings not as a result of occlusion. They suggested that

the depression was caused by inhibitory effects on the ganglion cells of the one group from the just before discharged cells of the other group and they supposed the occlusion to be small.

The occlusion between the two presynaptic systems and their effects on the msuculature and the blood flow of the colon when faradically stimulated have been investigated. A method for establishing occlusion has been worked out. A preliminary account of some of the experiments has already been published (OSCARSSON, 1954).

Methods.

The experiments have been made on cats narcotized with nembutal (40 mg/kg) or in a few cases with chloralose. The abdomen was opened and the colonic nerve was dissected free. This nerve forms a plexus around the inferior mesenteric artery and it is often not possible to claim that one special branch comes from one side only. Consequently it was in most cases necessary to dissect the hypogastric nerve and the inferior splanchnic nerves bilaterally. The nerves were kept in a pool of mineral oil in the abdominal cavity. A DC-amplifier and rectangular pulse stimulators were used.

In order to record the colonic tonus some experiments were made with intact colonic nerve and a balloon placed inside the colon and connected to a kymograph writer. In experiments for measuring the blood flow cats were heparinized (10,000 IU heparin per cat) and the total venous flow from the colon was passed through a polyethylene tube and the blood drops were counted. Every five or ten minutes the blood was transfused back into the femoral vein. In all experiments the ganglion was decentralized bilaterally.

Results.

1. The veratrine method for testing occlusion.

The veratrine method has been worked out in order to evade the difficulties in testing occlusion due to the contingency of inhibitory influences. Instead of using the refractoryness of the discharged cells as an indicator of occlusion, this has been tested after the complete recovery of the ganglion cells from excitability changes. This is possible by application of veratrine to the postganglionic nerve trunk, which causes a volley to be followed by a very long lasting negative afterpotential the effect of which can be detected in the shape and magnitude of a second volley in the same fibers.

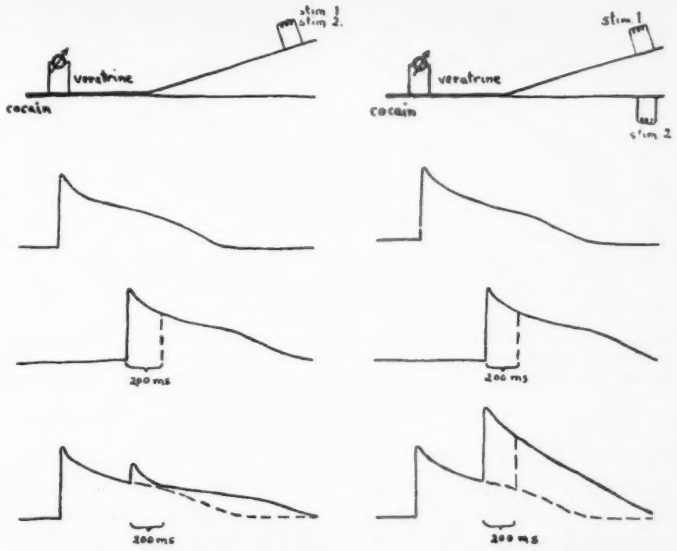

Fig. 1. The principles of the veratrine method. A model experiment on a branched splenic nerve from the cow. When the two simuli are given to the same branch (left) the negative afterpotential of the second volley is depressed whereas if they are given one to each branch (right) the negative afterpotentials are superposed. It has proved convenient to measure the negative afterpotential 200 milliseconds after the spike.

The method was tested on a branched splenic nerve from the cow. This nerve is composed almost exclusively of C-fibers. The preparation was kept at 36—38° C. Figure 1 illustrates the experiment. The recording electrodes are placed on the not branched end of the nerve, which for monophasic recording was treated with cocain at the electrode near the end. At the other electrode a solution of veratrine (1 : 100,000) was applied. This greatly increases and prolongs the negative afterpotential (GRAHAM and GASSER 1931, GRUNDFEST and GASSER 1938). During the very first part of the negative afterpotential of a volley the negative afterpotential of a second volley in the same fibers is almost completely depressed (JOB and LUNDBERG 1953). Figure 1, left, gives the results when both conditioning and testing stimuli are applied to one of the branches and consequently the impulses are propagated in the same fibers. The two upper curves show the action potentials when the stimuli are given alone, the lowest

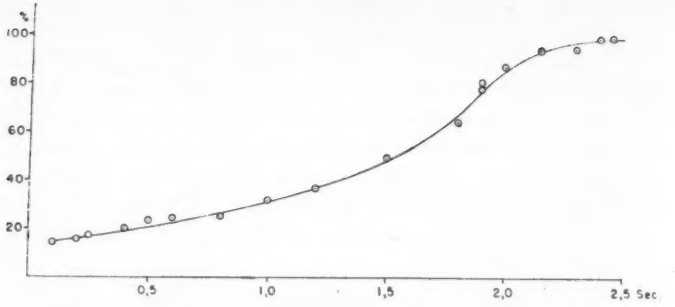

Fig. 2. The negative afterpotentials measured as shown in Figure 1, left, plotted against the time interval between the conditioning and testing spikes. Between 0.5 and 1.0 seconds the negative afterpotential is 20—31 per cent of the one not preceded by a conditioning volley.

one when they are given with a short interval. The spike and especially the negative afterpotential of the second volley are depressed to a high degree. The reduced negative afterpotentials are plotted against the time interval between the conditioning and testing spikes in Figure 2. The negative afterpotential is measured 200 milliseconds after the spike. The depression lasts about 2.5 seconds and is quite large during the first second.

If, on the other hand, the two stimuli are given one to each branch of the nerve (Fig. 1, right) the potentials are superposed and there is no depression of the negative afterpotential.

It is obvious that the method fulfils the designed purpose as the excitability changes in the ganglion cells under discussion last 600 milliseconds at the most. The method has also the advantage of enlarging the potentials through the syncronizing effect of the slowly falling negative afterpotential and by that making it possible to compare otherwise not measurable small and dispersed volleys. The following investigations have been rendered possible by the here introduced method.

2. Experiments on the reflex.
Comparison of different recovery curves.

Figure 3 shows the recovery curves from one experiment. The curves are drawn after plotting the magnitude of the spike set up in the colonic nerve by a testing stimulus preceded by a conditioning stimulus in per cent of the magnitude of the spike

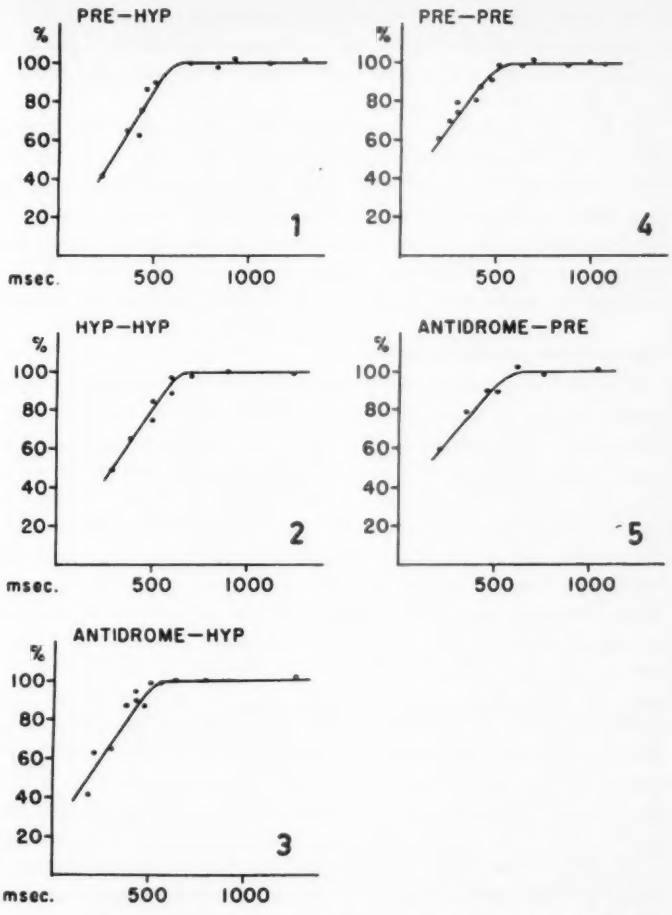

Fig. 3. Comparison of recovery curves from one experiment. The spike heights of the testing volleys are plotted against the time interval between the conditioning and testing volleys. Recording from the colonic nerve. Preganglionic (pre = inferior splanchnic nerves), hypogastric (hyp) and antidromic stimulation. In all cases there is a complete recovery after about 600 milliseconds.

without previous conditioning. In the first curve the conditioning stimulus is applied to the inferior splanchnic nerves and the testing one to the hypogastric nerve. The next two curves show the results when both conditioning and testing stimuli are given

to the same nerve either the inferior splanchnic nerves (4) or the hypogastric nerve (2). In the last two cases the inferior splanchnic nerves and the hypogastric nerve are tested when conditioned by a colonic nerve volley.

All the curves show a complete recovery after about 600 milliseconds and the curve form is approximately the same. Other experiments of the same type have given similar results. It is most easy to explain these depression curves as the result of refractoryness in the postsynaptic elements and thus to interpret the first curve as indicating occlusion between the fibers of the hypogastric nerve and those of the inferior splanchnic nerves.

Tests with the veratrine method.

In order to exclude the possibility that the depression was partly caused by some kind of inhibition not due to actual activation of the same ganglion cells the veratrine method was used. Figure 4 explains the method applied to this special problem. The upper curve corresponds to the first one in Figure 3 and gives the complete recovery time of the ganglion cells (450 milliseconds). The records used for plotting this curve were taken before veratrine treatment. The recordings for the three lower curves were made after veratrine had been applied to the colonic nerve at the proximal lead of the recording electrodes. . Conditioning and testing stimuli were now given to the inferior splanchnic nerves and the magnitude of the second negative afterpotential was plotted to give the next curve (2). After 600 milliseconds it was 28 per cent of the magnitude when not preceded by the conditioning volley. In the following curve (3) the stimuli are given as in the first one and the negative afterpotential is used for plotting the curve. After 600 milliseconds the value is 28 per cent. A control repetition of the second experiment again gave about the same value (30 per cent). This proves that the postsynaptic neurons that can be activated by stimulation of the hypogastric nerve also may be activated by stimulation of the inferior splanchnic nerves, and implies a complete occlusion. Other experiments of the same type have displayed an occlusion of 80—100 per cent.

The action potentials in the veratrinized colonic nerve resulting from stimulation of the inferior splanchnic nerves respectively the hypogastric nerve were found to be of approximately the same magnitude (see below). This finding together with the previous one of the high degree of occlusion could now be tested

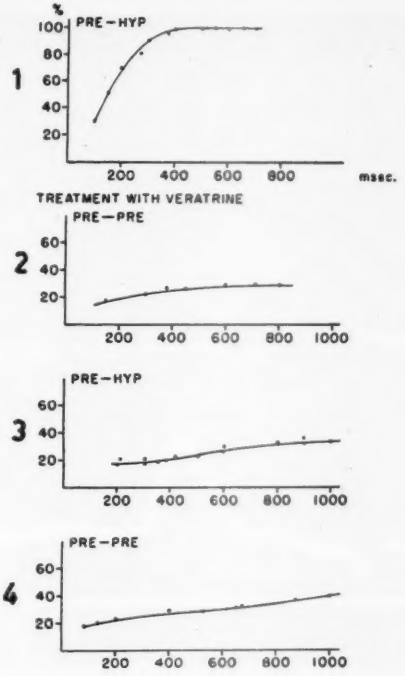

Fig. 4. An experiment for testing occlusion with the veratrine method. The upper curve corresponds to the first one in Figure 3. The three lower curves are based on recordings after veratrine treatment of the colonic nerve. See the text.

by measuring the occlusion when the conditioning stimulus is applied to the hypogastric nerve and the testing one to the inferior splanchnic nerves. This occlusion should be about 100 per cent. The veratrine method was used and an occlusion of 90—100 per cent was found (four experiments).

Comparison of action potentials.

The spike appearing in the colonic nerve after stimulation of the inferior splanchnic nerves is usually much more well-defined than the spike elicited by hypogastric stimulation. The latter is temporally dispersed and quite often has two maxima (Fig. 5). It is often very difficult or even impossible to compare the magnitudes of the volleys because of the small spike voltage and

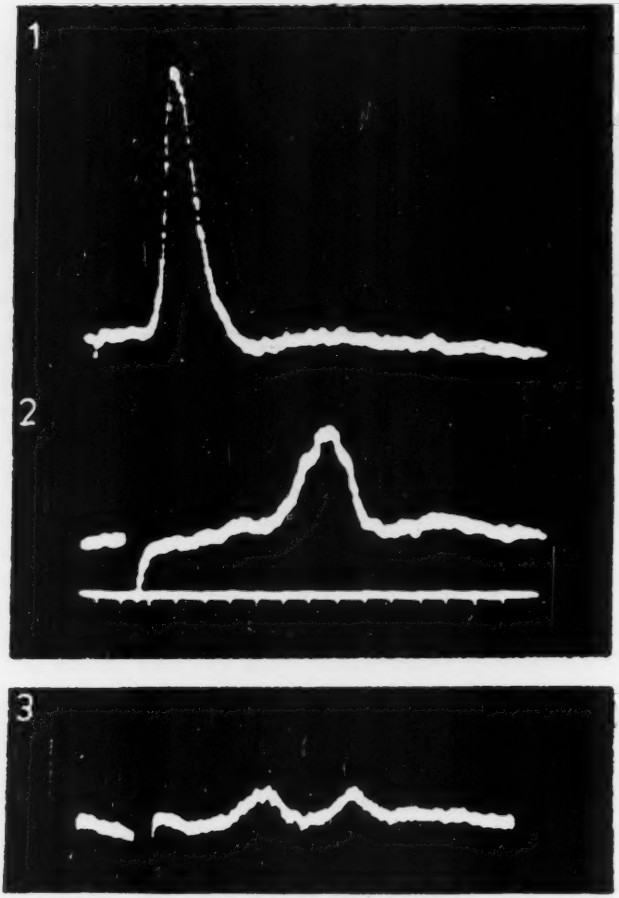

Fig. 5. Colonic recording. Stimulation of the ipsilateral inferior splanchnic nerves (1). Stimulation of the ipsilateral hypogastric nerve (2 and 3). 1 and 2 are records from the same experiment. Time marking 10 milliseconds.

great dispersion. That is, however, made possible by instead of comparing the spikes comparing the negative afterpotentials after veratrine application (Fig. 6). The magnitude of the volley from the hypogastric nerve usually has been 80—100 per cent of the volley given by preganglionic stimulation.

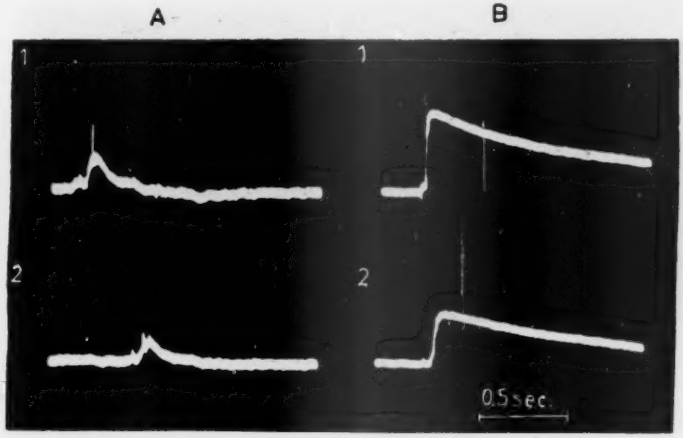

Fig. 6. Recording from the colonic nerve, A before and B after veratrine treatment. Stimulation of the inferior splanchnic nerves bilaterally (1). Stimulation of the ipsilateral hypogastric nerve (2).

In some of the experiments the hypogastric stimulation has given a smaller spike in the colonic nerve respectively a smaller influence on the colonic musculature and blood flow (see below) than preganglionic stimulation. This discrepancy has almost only been found in the first experiments of a type and especially in those connected with greater traumatizing. Later when the preparation technique was improved the results of preganglionic and hypogastric stimulation have been in good conformity. The earlier results are with all probability due to injury of the nerve fibers in the hypogastric nerve or the synapses between these fibers and the neurons in the colonic nerve, perhaps due to injury of the blood supply.

In a few cases it has been possible to compare the magnitude of the volley caused by supramaximal stimulation of the colonic nerve with volleys from the hypogastric and the preganglionic nerves. Figure 7 shows records from such an experiment. In this case the volley from the hypogastric nerve (2) was somewhat larger than that from the inferior splanchnic nerves (1). The negative afterpotential after hypogastric stimulation was 87 per cent and after preganglionic stimulation 80 per cent of that due to colonic stimulation.

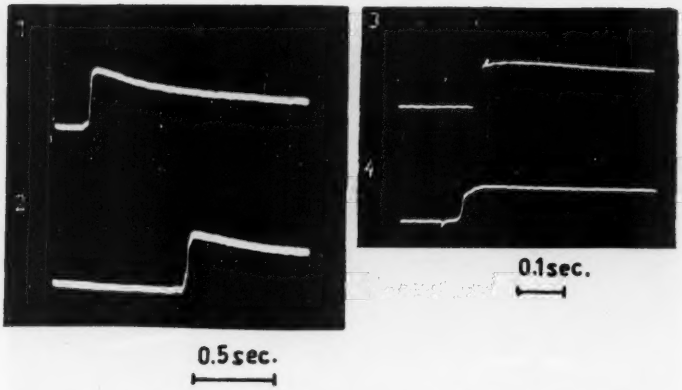

Fig. 7. Comparison of negative afterpotentials from the same experiment. Recording from the colonic nerve. Supramaximal stimulation of the inferior splanchnic nerves (1) and of the hypogastric nerves (2). Records 3 and 4 are taken with lower amplification than 1 and 2. Supramaximal stimulation of the colonic nerve (3) and of the inferior splanchnic nerves (4).

Also in the other experiments of this type the volley after colonic stimulation was somewhat greater than the volley caused by preganglionic or hypogastric stimulation. This may be due to the existence of centripetal fibers with their trophic centers in the periphery, similar to those found in the hypogastric nerve. The occurrence of such fibers in the colonic nerve has been reported by KUNTZ and SACCOMANNO (1944). After degeneration following decentralization (cutting both the inferior splanchnic nerves and the hypogastric nerves) of the inferior mesenteric ganglion histological sections showed undegenerated nerve endings in the ganglion. Nerve fibers remained intact in the distal segments of the branches of the colonic nerve after degeneration of all centrifugal fibers.

Effect on the colonic musculature.

Figure 8 is a kymogram of the activity and tonus of the colonic musculature. Without stimulation there are rhythmic contractions with a frequency of about 30 per hour. The hypogastric nerve is faradically stimulated at "hyp" causing cessation of the rhythmic activity and a decrease of the tonus. After stopping the stimulation there is a slow increase of the tonus and after a period of irregular contractions a restoration of the previous

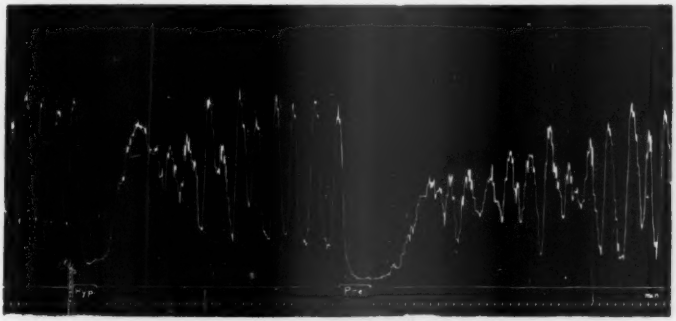

Fig. 8. A kymogram of the intracolonic pressure, showing the effect on rhythmicity and tonus when faradic stimulation is applied to the hypogastric nerve (hyp) and to the inferior splanchnic nerves (pre). Stimulation with a frequency of 15 per second.

rhythmicity. Stimulation of the inferior splanchnic nerves gives a similar response, in this experiment somewhat stronger than that from the hypogastric nerve.

In other experiments the effects were approximately equal but in many there was a slightly greater effect when stimulating the inferior splanchnic nerves.

Effect on the blood flow.

The effect on the blood flow has been investigated in a few experiments. As a control the colonic tonus has been recorded at the same time. The colonic blood flow was measured by the drop-counting method. It was found that the blood flow was decreased by stimulating the hypogastric nerve or the inferior splanchnic nerves and that the reduction in all cases was parallel with the effect on the musculature. The blood flow was normally fairly constant and the spontaneous contractions did not influence it appreciably, neither was it influenced by increasing or decreasing the intracolonic pressure with 12 cm of water. The blood flow changes therefore seems to be due to a direct effect on the blood vessels. Figure 9 A and B illustrates an experiment of this type.

Experiments to establish the physiological stimulus.

Distension of the distal part of the transected colon or of the anal sphincters has given no appreciable effect as judged from

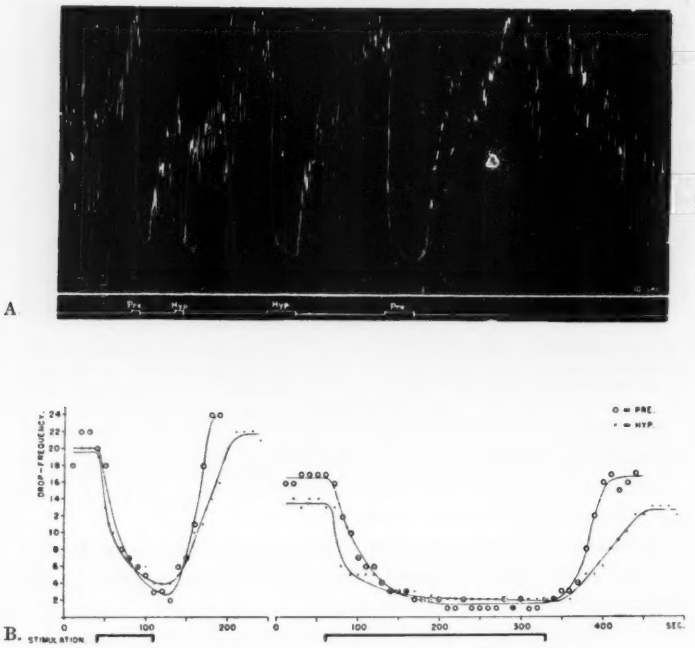

Fig. 9. The effect of faradic stimulation of the inferior splanchnic nerves (pre) and of the hypogastric nerves (hyp) on the intracolonic pressure (A) and on the colonic blood flow (B) simultaneously recorded. The blood drops are counted and plotted for every ten seconds. Stimulation time 70 respectively 270 seconds. Stimulation with a frequency of 15 per second.

recordings of the intracolonic pressure. Neither has any effect been observed after electrical stimulation of the external sphincter, injury to the perianal region, or instillation of hypo- or hyperosmotic solutions in the distal part of the colon. (These experiments were made when the hypogastric-colonic reflex was thought to be a local mechanism causing relaxation of the colonic musculature only.)

Discussion.

The volley in the colonic nerve elicited by hypogastric stimulation presents a recovery curve when preceded by antidromic activation of the ganglion cells that is very alike the curve re-

sulting from preganglionic conditioning of the inferior splanchnic nerves. Similar curves are also obtained when the two stimuli (conditioning and testing) are given to the same presynaptic nerve. This conformity of the curves makes it very probable that the depression in all cases is caused by refractoryness of the ganglion cells and thus suggesting a high degree of occlusion.

This was definitely proved by experiments with the veratrine method by which it is possible to establish occlusion after the disappearance of the excitability changes of the ganglion cells. This method has also proved particularly useful for estimation of how large a part of the ganglion cell pool that could be activated from the presynaptic fibers in the hypogastric nerve. Without veratrine the spike is so dispersed and small that comparison of its area with that of the spike obtained on stimulation of the inferior splanchnic nerves or the colonic nerve meets with great difficulties. By veratrinizing the colonic nerve these difficulties are avoided and the magnitude of the negative afterpotential gives an approximative measure of the number of neurons activated when compared with the negative afterpotential obtainable on direct stimulation of this nerve. In view of the earlier findings by JOB and LUNDBERG (1952) it was a surprise to find that in a number of experiments as many as 80—100 per cent of the cells that were activated from the inferior splanchnic nerves also were activated by stimulation of the presynaptic fibers in the hypogastric nerve.

This finding undoubtedly increases our interest as to the physiological significance of the reflex under discussion. However, there is hitherto no indication of what constitutes the adequate stimulus for the presynaptic neuron. The parallel effects on muscular tonus and blood flow suggest a more general effect than could be expected from a mechanism subserving for instance the motor integration of colon and rectum. There is also some indication that this activation of sympathetic neurons from the periphery may be a general phenomenon in prevertebral ganglia. Similar reflexes have recently been found in the superior mesenteric and the coeliac ganglia (HOLMQVIST 1955). The findings by KUNTZ and SACCOMANNO (1944) point in the same direction. Their experiments were performed after degeneration following decentralization of the inferior mesenteric ganglion. The colon was divided at the level of the inferior mesenteric artery and distension of the distal segment of the colon or electrical stimulation of its

nerves, resulted in reflex inhibition of the muscular rhythmicity and tonus of the proximal segment. Similar observations were made on the coeliac ganglion and corresponding gut portions.

Summary.

The organization of two presynaptic systems (in the inferior splanchnic nerves and in the hypogastric nerve respectively) of the colonic nerve neurons in the inferior mesenteric ganglion of the cat has been investigated. Electrophysiological experiments have demonstrated extensive convergence of the two systems on postganglionic neurons (occlusion 80—100 per cent). The great majority of the colonic nerve neurons could be discharged from either of the two presynaptic systems as measured with the veratrine method worked out for this purpose. Faradic stimulation of either of the two presynaptic systems has given qualitatively and quantitatively similar changes in the muscular tonus and the blood flow of the colon.

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From the Institute of Physiology, University of Upsala, Sweden.

Transmucosal Migration of Water, Sodium, Chloride and Hydrogen Ions in Rabbit Ureters.

By

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It is generally assumed that the urine formed by the kidney is transported through the urinary tract without any substantial change in composition. JOHNSON, CAVERT, LIFSON and VISSCHER (1951) showed, however, that the bladder mucosa in dogs allows a fairly rapid exchange of water between the urine and the blood. Their experiments indicated also that a small net transport of salt across the barrier may take place, the direction of transport being from the high concentration to the low one.

No experiments seem to have been performed concerning the transport of water and solute matter across the mucosa of the ureters. The present work is an attempt to show that such a transport takes place, and that its magnitude is such that, at least for some purposes, it cannot be neglected.

Methods.

General experimental arrangement. Healthy albino rabbits, weighing about 2 kg, were used throughout. They were anaesthetized with Nembutal (Abbott). The ureters were exposed at both their ends and were cannulated with polyethylene tubings just below the pelvis and just above their entrances into the bladder. The proximal polyethylene tube was connected to an apparatus for continuous injection (ÖBRINK 1948), and almost the whole ureter could thus be perfused. The perfusion rate varied in the different experiments between 16 μl/minute and 36 μl/minute. The experiments lasted for about 1—3 hours. The

following solutions were used for perfusion: in tracer experiments, the tracer was added to a Krebs-Ringer-bicarbonate solution (COHEN 1945). In experiments on net salt transfer, two solutions were used, one containing about 15 millimols NaCl per litre and the other about 300 millimols NaCl per litre. In experiments on hydrogen ion transfer, the perfusion fluid contained about 100 millimols NaCl per litre and about 22 millimols KH_2PO_4 per litre.

As a rule, the liquid emerging from the distal tube was quite clear, but in a few experiments on net salt transfer, a small amount of blood could be detected. When the percentage of blood in the fluid, as estimated from haematocrit and haemoglobin determinations, was more than 1 % (it never exceeded 2 %), corrections were made for this contribution both as regards salt concentration and volume changes.

Analytical Methods.

Deuterium oxide. Samples from the perfusate were taken at certain time intervals (see figure 1) and distilled according to GARBY (1955). Thereafter, the content of deuterium oxide was determined by the method of LINDERSTRÖM-LANG, JACOBSEN and JOHANSEN (1938). The notation D_2O is used throughout in this paper though in dilute solutions essentially HDO molecules are present.

Radioactive chloride. Cl^{36} was used. It was obtained from the Radiochemical Centre, Amersham, England, and, when used, contained no activity from other isotopes. 0.1 ml of the samples to be analyzed were pipetted into small aluminium dishes covered with filter paper. One drop of a 50 % glucose solution was added and the preparation was dried at about 100° C. A GEIGER-MÜLLER tube was used for detection. At least 2,500 impulses were counted and the activity was about 400—500 counts per minute.

Chloride. The chloride concentration in the different samples of 0.1 ml was determined by electrometric titration.

Sodium. The sodium concentration in the 0.1 ml samples was determined by flame photometry, a Perkin Elmer flame photometer with internal lithium standard being used.

pH. The hydrogen ion concentration was measured on samples of about 1 ml by aid of a glass electrode arrangement. All measurements were carried out at room temperature and the solutions were allowed to equilibrate with the CO_2-tension of the air.

Results.

Tracer experiments. Two experiments were performed. To the Krebs-Ringer-bicarbonate solution used as perfusion fluid D_2O was added to give a concentration of about 7 % (w/w). Furthermore, Cl^{36} was added to give approximately 450 counts per minute per 0.1 ml.

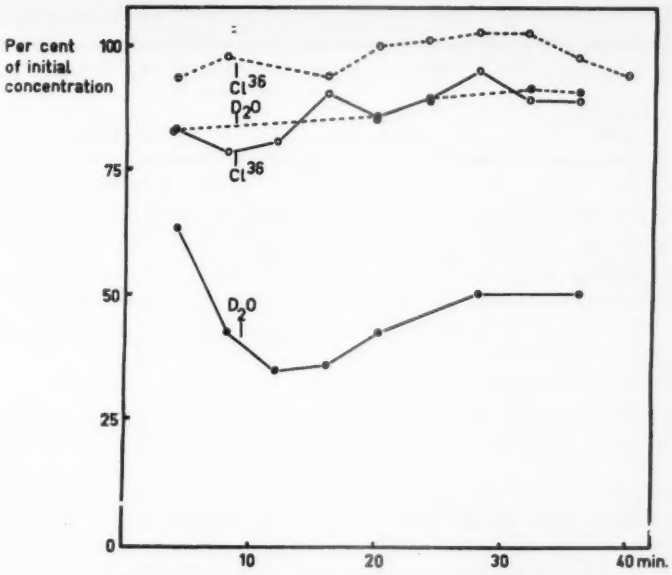

Fig. 1. This figure shows the time course of the concentrations of D_2O and Cl^{36} in the fluids having passed the ureters, expressed as per cent of initial perfusion fluid concentration. The perfusion was started at zero time. The full lines correspond to the experiment with the low perfusion rate and the dotted lines to that with the high perfusion rate (see text).

The results from the experiments is shown in figure 1. It is evident that, when a steady state was reached, the concentration of D_2O and Cl^{36} was lower in the fluid emerging from the ureter than that entering, the concentration of D_2O being about 50 % and that of Cl^{36} about 90 % of the initial perfusion fluid in one experiment. (The high value in the beginning of this experiment was due to a slight experimental mishap: the first few drops were forced through the ureter by hand at a considerably increased velocity.) The length of the perfused part of the ureter was in this case about 7 cm, and the perfusion rate was 30 μl/minute. In the other experiment, the perfusion rate was 36 μl/minute and the length of the ureter about 5 cm. In this case the D_2O concentration in the outlet fluid at steady state was about 90 % of the inlet fluid and the Cl^{36} concentrations did not differ significantly from each other.

Table 1.

Composition of perfusion fluid		Per cent change in concentration and volume after perfusion at high rate ~ 32 µl/min.				low rate ~ 16 µl/min.			
		Experiment				Experiment			
		1	2	3	Mean	4	5	6	Mean
NaCl ~ 300 mM	Cl^-	— 44	— 8	— 13	— 22	— 44	— 18	— 14	— 25
	Na^+		— 16	— 20	— 18		— 27	— 22	— 24
	Vol.	+ 0.2	0.0	+ 0.7	+ 0.3	— 6.8	— 1.5	— 6.8	— 5.0
		7	8	9	Mean	10	11	12	Mean
NaCl ~ 15 mM	Cl^-	+ 32	+ 20	+ 19	+ 24	+ 6	+ 21	+ 36	+ 21
	Na^+		+ 18	+ 51	+ 35		+ 35	+ 58	+ 47
	Vol.	0.0	+ 0.2	+ 3.5	+ 1.2	+ 0.3	— 5.0	— 1.1	— 1.9

Experiments on net transfer of sodium, chloride and water. The results from 12 experiments are seen in table 1. It is evident from this table that a considerable change in concentration of both chloride ions and sodium ions during the passage through the ureter took place. The 300 mM solution lost about 20 % of its content of chloride and sodium and the 15 mM solution gained in chloride concentration by about 20 % and in sodium concentration by about 40 %.

In these experiments, changes in volume during perfusion were also investigated. The total fluid volume emerging during a certain time interval was measured and, as the rate of volume delivery of the injection apparatus was known, any net change in volume could be recorded. The results are seen in table 1. The changes in volume during perfusion were small, generally less than 5 %.

Experiments on hydrogen ion transfer. Two experiments were performed with a perfusion rate of 16 µl/minute. The buffer solution used for perfusion had a pH of 4.63, the fluid emerging from one ureter had a pH of 5.75, and that from the other ureter a pH of 5.65. After saturating these fluids with air containing about 5 % CO_2, the pH was lowered by about 0.1 unit.

The perfusion fluid contained less than 0.1 % of blood after the passage through the ureters. The relation between the buffer

capacities of the blood and the perfusion fluid was such that the addition of 3 parts of blood to 1,000 parts of perfusion fluid gave an increase in pH of about 0.15 units.

Discussion.

The experiments on tracer disappearance from the perfusion fluid show that the resistance to water and chloride transport across the mucosa in the ureters is not infinitely large. Therefore, if driving forces for these components should exist, a net transfer of water and chloride should occur. The direction of such a net transfer would be in the direction of the electro-chemical potential gradient across the barrier if "active" forces do not exist. As nothing is known about the driving forces in the present system, the tracer experiments cannot be used for an evaluation of the direction and rate of the net transports.

The experiments on net transfer of sodium and chloride show, however, that concentration gradients of sodium and chloride of the magnitude used here work as effective driving forces, the direction of transport always being from the high concentration to the low one. The magnitude of the net transports of ions is comparatively larger than that of the water transport which is to be expected. A concentration gradient for water is certainly present, but, as the barrier is not impermeable to the existing solutes, the theoretical van't Hoff pressure is not reached (cf. STAVERMAN 1951). Of course, if most of the solute components consist of ions or molecules that slowly or not at all penetrate the membrane, larger "osmotic" effects might be expected.

The profound change in pH of the fluids during perfusion may have several explanations. The most probable one is that hydrogen ions have left the lumen by way of a cation exchange mechanism. However, an exchange of bicarbonate ions or hydroxyl ions in the opposite direction cannot be excluded. The amount of CO_2 entering the ureter during perfusion (and leaving the solution during contact with atmospheric CO_2-tension) contributes very little to the change in pH since the pH of the solution did not change more than 0.1 unit after re-saturating it with air containing about 5 % CO_2.

The question may be raised whether the conditions in the present experiments are physiological or not. It is evident that

the magnitude of both the exchange and the net transport of matter as shown in the present experiments may depend on the rate at which the fluid travels along the ureters and the extent of mixing within the lumen. If, at "diureses" of the magnitude used here, the passage through the ureters is essentially intermittent during physiological conditions, the linear rate of flow undoubtedly becomes larger and the time of contact between the fluid and the epithelium correspondingly shorter. At the same time, however, the mixing must be assumed to become more intensive. It is not known whether any one of these two processes controls the rate of transmucosal transport. Furthermore, even if intermittent flow exists, a certain, in all probability not negligible, amount of fluid is always present in the lumen. This fluid will undoubtedly make any estimation of a "contact time" based on the frequency and velocity of peristaltic waves much too small.

Therefore, the present experiments cannot be used to estimate with certainty the magnitude of the transmucosal migration of different components during physiological conditions. It may be assumed, however, that they give a fair amount of information as to expected magnitudes.

Summary.

The change in composition of fluid perfused through rabbit ureters *in vivo* was investigated. It was shown that

1. labelled water and chloride can leave the ureter through the mucosal barrier;

2. during certain conditions, a net transport of sodium and chloride through the mucosa takes place, the direction being along diminishing concentration gradients;

3. moderately hypo- and hypertonic sodium chloride solutions evoke very small volume shifts during passage;

4. hydrogen ions may easily disappear during the transport along the ureter, presumably by way of transmucosal transport.

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Innervation Zone and Propagation Velocity in Human Muscle.[1]

By

FRITZ BUCHTHAL, CHRISTIAN GULD and POUL ROSENFALCK.

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Motor unit responses in a muscle vary greatly with respect to duration, amplitude and shape (BUCHTHAL, GULD and ROSENFALCK 1954). Among factors which might be responsible for this variation are the distribution of motor end plates in the motor unit and the propagation velocities over the different muscle fibres. In a previous study, the propagation velocity of the action potential over electrically stimulated muscle fibres was shown to vary within a narrow range (4.02 m/sec. S. D. = 0.43 m/sec.) (BUCHTHAL, GULD and ROSENFALCK 1955). In voluntary contraction, DENSLOW and HASSETT (1943) found propagation velocities ranging from 1.3 to 12.5 m/sec. In their experiments no consideration was taken as to the site of innervation relative to the recording electrodes and no attempt was made to ascertain that the different electrodes led off potentials from the same fibres.

The discrepancy between velocities found in electrically and in voluntarily activated muscle fibres made it desirable to reinvestigate propagation velocity during voluntary activation utilizing the techniques employed in the determination of propagation velocity with electrical stimulation and with more precise knowledge as to the site of innervation.

[1] Aided by a grant from the Michaelsen Foundation, Copenhagen, and the Muscular Dystrophy Associations of America, Inc., New York.

In the present study, the zone of innervation was estimated both anatomically and by action potential recordings. Anatomically, the distribution of the terminal nerve branches was studied. Electrophysiologically, the zone of innervation for the individual motor unit was determined as the region in which potentials from a given motor unit began simultaneously.

Finally, an attempt was made to account for the narrow range of velocities in terms of the conducting properties of the medium surrounding the active fibres in situ.

Methods.

All measurements were performed on the human brachial biceps. The ten subjects were 20 to 24 and 47 years of age without signs or symptoms of neuromuscular disease. Records were taken from the same motor unit with up to six concentric electrodes during weak voluntary effort; and during electrical stimulation through a bipolar electrode inserted into the distal region of the muscle. The technique of stimulation has been described (BUCHTHAL, GULD and ROSENFALCK 1955). A three-channel DISA electromyograph was used for recording. The concentric recording electrodes had a core diameter of 0.1 mm and a tip angle of 20°. The properties of the differential amplifiers and the electrodes have been described in detail (BUCHTHAL, GULD and ROSENFALCK 1954). When it was desired to record with more than three electrodes, one electrode was kept as reference in one of the channels, while the other electrodes could be switched at will to the other two channels. A 50 μsec. square wave pulse was introduced in the three channels of the electromyograph in order to obtain a simultaneous signal on the three cathode ray tubes. In experiments with electrical stimulation this signal was simultaneous with the stimulus. The potentials were recorded with single sweeps on photographic paper moving at a speed of 5 cm per second. The sweep velocity was one mm per msec. and the frequency two to four per second.

The arrival time of the action potential was determined by measuring the time interval between the marking signal and a well defined point on the action potential at the different recording points. In determinations of the innervation zone, measurements were referred to the onset of the potential. Measurements of propagation time were referred to the point at which the steep deflection from positive to negative intersects the base line. The presence of such a steep deflection in the potential indicates that the recording electrode is situated in the immediate vicinity of an active fibre, as judged by the fact that over 0.5 mm the steep deflection of the action potential had diminished to one fifth or less (to be published). In experiments with electrical stimulation the steep deflection was searched for by small displacements of the recording electrodes in the transverse plane of the muscle.

The distance between the tips of the recording electrodes within the muscle was determined as accurately as possible by correcting the electrode distance measured on the skin for the depth and angle at which the electrodes were inserted into the muscle.

Histology:[1] Since it was impossible to make total longitudinal sections of the adult muscle, such sections were made of the whole brachial biceps of a newborn infant. Findings on this muscle were confirmed on the short head of the adult brachial biceps by serial longitudinal sections of adjacent regions, 2 cm each in length (Fig. 2). The muscles were fixed in four per cent neutral formaline saline solution, embedded in paraffin, sectioned at 10 μ intervals and stained for neurofibrils by the DAVENPORT method (1930). The sections were examined for the finest nerve branches. At the level where these had a diameter of only about 1 μ, they occurred in small "brushes" each containing a mean of 12 fibres (S. D. = 6 fibres). Whenever a fibre of these brushes could be followed to its entrance in a muscle fibre, the end plate was situated within a maximal distance of 2 mm.

Results.

1. The zone of innervation.

The zone of innervation was estimated both histologically and by action potential recording.

a) *Distribution of the terminal nerve endings along the brachial biceps.*

Each head of the brachial biceps had its narrow band of motor nerve terminals situated in the proximal third of the total length in the short head and in the distal third in the long head. Fig. 1 shows the site of the terminal nerve endings in four superimposed consecutive sections from the short head of the biceps of a newborn infant. The counts of terminal nerve branches from four different regions of the short head of the biceps in the adult are

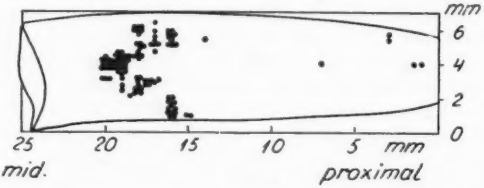

Fig. 1. Site of terminal nerve endings in the short head of the brachial biceps of a newborn infant. Four consecutive longitudinal sections superimposed. 0 mm corresponds to the proximal tendon end.

[1] We acknowledge with thanks the help of ERNA CHRISTENSEN, M. D., Laboratory of Neuropathology, Institute of Pathology, University of Copenhagen, who was responsible for the histological preparations.

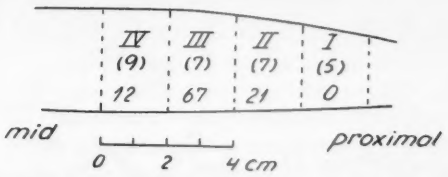

Fig. 2. Incidence of terminal nerve endings in four adjacent regions (I—IV) of the short head of the brachial biceps in the adult. The lower figures indicate the relative incidence of terminal nerve endings in the different regions. The figures in brackets denote the number of horizontal sections examined.

given in Fig. 2. Sixty-seven per cent of all terminals were localised within area III, a band in the proximal third of the muscle comprising two cm, *i. e.* ten per cent of its total length (length of muscular portion 160—210 mm, 17 muscles).

b) *Innervation zone as determined by action potential recording.*

When the motor unit potential was recorded at different points within the muscle, a zone was found corresponding to the anatomical innervation zone in which the onset of the potentials was abrupt and simultaneous within 0.5 msec. This simultaneity indicates that the propagation time over the efferent nerve fibres to the different muscle fibres of a given motor unit is identical within 0.5 msec. Records from the innervation zone of two different motor units (A, B) are illustrated in Fig. 3. Ordinarily the initial phase of the potential was positive (Fig. 3, A_1, A_2, B_3, B_4, B_5), but by very small displacements of the electrode in the transverse plane of the muscle the onset of the potential could be changed from a small positive to a steep negative deflection (Fig. 3, A_2, A_3, B_1, B_2). In the center of the region such potentials with a steep negative onset were found readily; in the periphery of the zone only after searching. The fact that it is possible in this region to obtain action potentials with a steep negative onset by small displacements of the electrodes (less than one mm) shows that active fibres are situated near the recording electrodes. The fact that the potentials have a simultaneous onset on the different electrodes shows that the leading off occurs near the site of initiation of the potential. The finding of a purely negative onset of the action potential points in the same direction. It must be concluded that the site of innervation for different muscle fibres of the motor unit must be spread over the region in which an *abrupt, simultaneous* onset of the motor unit potential

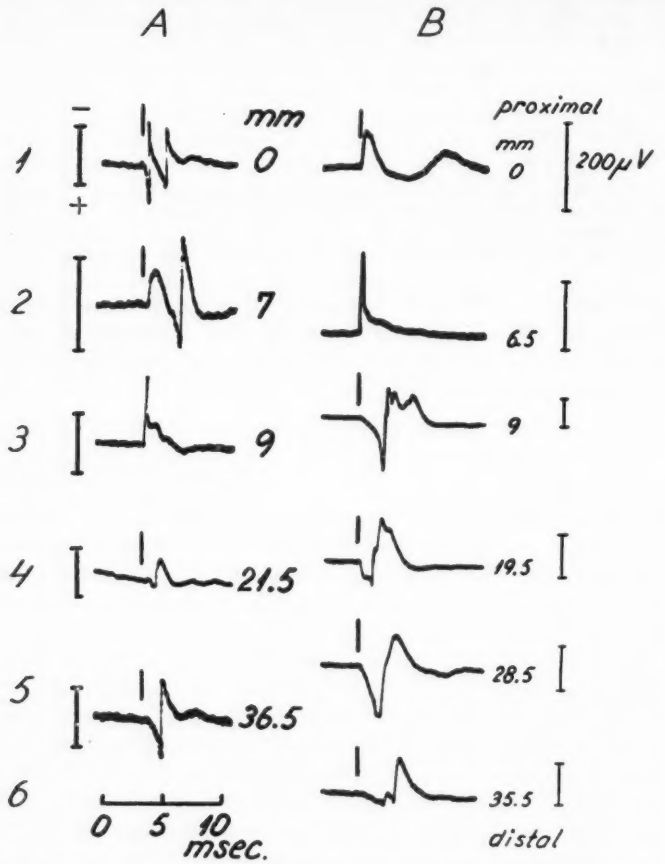

Fig. 3. Two motor units examined in different points of the innervation zone along the longitudinal axis of the brachial biceps. The position of the electrodes is given to the right of the records. Simultaneity is indicated by the vertical lines. Note the initial abrupt negative deflection in A_2, A_3 and B_1, B_2.

was found. The maximum extension of this region was 40 mm corresponding to the maximum width of the zone of innervation for the individual motor unit. The agreement between anatomical and electrophysiological findings indicates that the innervation zone can be determined by action potential recordings in other muscles as well.

2. Propagation velocity during voluntary effort.

When two electrodes were placed in the same motor unit distal to the innervation band along the longitudinal axis of the muscle, their spike potentials were separated in time. From this time interval and the distance between the electrodes, propagation velocities were calculated which varied between 2.7 m per sec. and such unreasonably high velocities as 20 to 100 m per sec. Moreover, recording with three electrodes distal to the zone of innervation, the propagation velocity found with the distal and the mid electrode generally differed from that determined with the mid and the proximal electrode. These findings suggest that there was a considerable chance of recording spikes from different fibres of the motor unit on the different electrodes. The apparent large range in "propagation velocity" is then explicable on the basis of the relatively wide zone of innervation described above.

It was, therefore, necessary to ensure that the three electrodes actually picked up potentials from the same fibres. For this purpose, three recording points along the longitudinal axis of the muscle were identified by electrical stimulation as being situated near the same fibres (BUCHTHAL, GULD and ROSENFALCK 1955).

The voluntarily released action potentials recorded in these points ordinarily contained only a single steep deflection (from positive to negative), so that there was no doubt as to what point of the potentials should be used for the determination of the arrival time of the impulse. A final assurance that the three electrodes recorded from the same fibres was the finding of a rectilinear relationship between arrival time and electrode distance for both electrically and voluntarily released potentials (Fig. 4). The rectilinear relationship obtained for half of the units tested. The activity of up to five different motor units could be identified at the three points.

The mean propagation velocity for voluntarily initiated motor unit potentials was 4.7 ± 0.1 m/sec. (36.5° C., Table 1), with a maximum variation within the individual muscle of 1.3 m/sec. The mean propagation velocity found with electrical stimulation was 4.1 ± 0.15 m/sec. This value is identical with the previously found value ($4.02 + 0.13$ m/sec., BUCHTHAL, GULD and ROSENFALCK 1955). The action potentials from voluntarily activated fibres had thus a significantly higher propagation velocity than found with electrical stimulation.

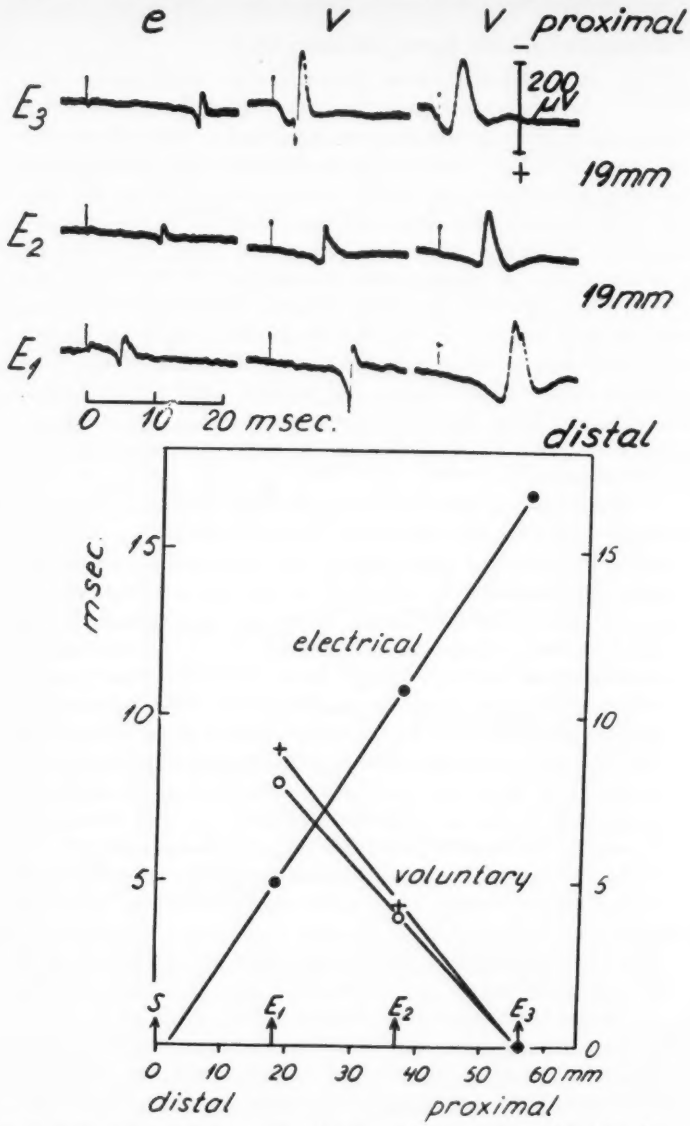

Fig. 4. Propagation of action potentials released during electrical (e) and voluntary (v) activity.

Upper records: Action potentials led off at three different points (E_1, E_2, E_3) along the longitudinal axis of the brachial biceps. Electrode distances E_1, E_2 = 19 mm, and E_2, E_3 = 19 mm.

Below: Arrival time of the above spike potentials plotted as a function of electrode distance. Electrically and voluntarily released activity recorded in the same points. Electrical stimulation through electrode S placed in the distal part of the muscle causes propagation from distal to proximal; voluntary activation causes propagation from proximal to distal. Propagation velocities: V_e = 3.2 m/sec., V_v = 4.3 m/sec. and 4.8 m/sec.

Table 1.

Propagation velocity at voluntary effort and at electrical stimulation. Brachial biceps; 36.5° C.

Subject	Age	Velocity in m/sec.	
		Voluntary activation	Electrical stimulation
F. E.....	21	5.3 5.1 4.7 4.6	4.2
P. R.....	20	5.9 5.9 5.1 4.6 4.6	4.7
K. U.	22	4.8 4.7 4.6 4.3 4.2	4.2
H. A.	21	4.3 4.2 3.7	3.9
F. B.....	47	5.4 5.3 5.1 4.7 4.7 4.6 4.2 4.1 4.1	4.5 3.8 3.4
mean value		4.72 ± 0.1 m/sec. S. D. = 0.54 m/sec.	4.10 ± 0.15 m/sec.

Discussion.

The statement is frequently found that especially in long muscle, the fibres of a motor unit are arranged partly in series and partly in parallel within the muscle (ADRIAN 1925, COOPER 1929, BRODAL and REFSUM 1942, VAN HARREVELD 1946, DENSLOW and GUTENSOHN 1950, FEINDEL 1954). This has not been found to be true for the brachial biceps in man. Our previous findings with electrical stimulation (BUCHTHAL, GULD and ROSENFALCK 1955) of

a constant velocity over a distance of more than 100 mm, has shown that the individual fibres pass uninterrupted over at least this length of the muscle. Nor do the anatomical studies give an indication of a serial arrangement of fibres in the brachial biceps. The narrow innervation zone (67 per cent of nerve terminals within 10 per cent of the total length of the muscle) observed in this study agrees with findings of COËRS (1953) and would support the electrical evidence of uninterrupted passing of the fibres from one tendon end to the other. COËRS (1953) has used a more direct method (KOELLE 1950) for the determination of the site of the motor nerve endings and has found that they are localised near the middle of the fibre. In the case of the brachial biceps, he found the end plate region to consist of two closely adjacent slightly concave bands. The extent of this area is not given in COËRS report.

The simultaneous onset of the motor unit potential within a certain region of the muscle might have been caused by volume conducted currents from a distant generator. The steep decrease in spike amplitude over a distance of less than one mm (to be published) excludes this possibility and, therefore, the recording electrode must have been situated very close to the active fibres. This entails that whenever potentials are recorded from a motor unit which have a simultaneous abrupt onset, be it negative or positive, they are led off within the zone of innervation. Thereby it is possible electrophysiologically to determine the extension of this zone for the individual motor unit in different muscles. The simultaneity of the onset of the potentials in the innervation zone indicates also the identity between the anatomically and the electrophysiologically determined motor unit. If the responses of an electrophysiological motor unit were evoked by two or more ganglionic cells, systematic deviations from the synchrony of the onsets of the potentials within the innervation zone would have been expected on account of differences in the propagation time from the ganglionic cells to the muscle fibres.

The present study has shown that voluntarily released action potentials are propagated with velocities which vary within a narrow range. The large variation which obtains without precautions with respect to the site of the electrodes relative to the zone of innervation and to placement near the same fibres is no expression of a true variation in propagation velocity. It is an expression of different starting points of the impulse.

It could have been expected that the *differences in action potential duration* found during voluntary effort in various points of a muscle could be accounted for in part by differences in propagation velocity. However, the present finding of an extremely narrow range of propagation velocities within the muscle leaves this a factor of only secondary importance. Differences in length of the terminal nerve branches can not be a contributory factor, since potentials showed a practically simultaneous onset in the different regions of the innervation band. Hence, the action potential durations must be explained in terms of the extent of the innervation zone. An innervation zone of 40 mm could with a propagation velocity of 4.7 m per second account for differences in propagation time for the different fibres of a motor unit of 8.5 msec. With a 2—3 msec. duration of the action potential of the individual muscle fibre, and with an additional 3 msec. arising from dispersion of the potential during propagation in the region outside the innervation band (BUCHTHAL, GULD and ROSENFALCK 1955), action potential durations of 10—14 msec. could be obtained. Within the zone of innervation shorter action potential durations occur. In the center of this region the differences in propagation time for different fibres of the motor unit would with a 40 mm innervation zone amount to 4 msec., corresponding to an action potential duration of 6 msec. Even shorter durations would obtain if the recording electrode were placed in such a way that it picked up the potential from part of the motor unit only.

Propagation velocity and external resistance.

Propagation velocity in nerve and muscle has been interpreted in terms of local circuits arising in a cable-like structure. For fibres immersed in a large volume of conducting fluid, the external resistance can be disregarded and according to theory the propagation velocity varies with the square root of the fibre diameter (OFFNER, WEINBERG and YOUNG 1940, KATZ 1948, HODGKIN 1954).

With muscle fibres in Ringer's solution there is an increase in propagation velocity with increasing diameter (HÅKANSSON 1954). Therefore, our finding of a narrow range of propagation velocities in situ is apparently inconsistent with the threefold variation in fibre diameter within the muscle (BUCHTHAL, GULD and ROSENFALCK 1955).

Under conditions where the external resistance cannot be disregarded, the velocity (V) is

$$V = a \sqrt{\frac{D}{1 + \frac{r_e}{r_i}}} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where D represents the fibre diameter, r_i the internal, r_e the external longitudinal resistance per cm, and a is a factor independent of D. r_i varies in inverse proportion to the area of the fibre, *i. e.* to the square of the diameter D. r_e is assumed to be inversely proportional with the surface of the fibre, *i. e.* with the diameter of the active fibre. Hence

$$\frac{r_e}{r_i} = kD \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where k is a factor independent of D. Consequently, the variation of the propagation velocity V with the fibre diameter is determined by:

$$V = \frac{a}{\sqrt{\frac{1}{D} + k}} \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Thus, with increasing diameter D, the propagation velocity approaches a maximum value, $V_{max.} = \frac{a}{\sqrt{k}}$, (Fig. 5). At fibre diameter $D = \frac{1}{k}$ the propagation velocity amounts to 70 per cent of the maximum. Therefore, a variation in velocity similar to that found in the present experiments (about 30 per cent) might be ascribed to the influence of the external resistance provided the minimum fibre diameter $D_{min.}$ satisfies the condition: $\frac{1}{D_{min.}} = k$, *i. e.* if the external resistance is equal to or larger than the internal resistance. A high external resistance in situ would arise if the inactive fibres act as insulators. Whether they act as insulators is estimated by comparing the impedance of the interspace with that of a muscle fibre to transverse currents.

For simplicity, let the muscle fibres be represented as rods one cm long with a quadratic cross section and side length D

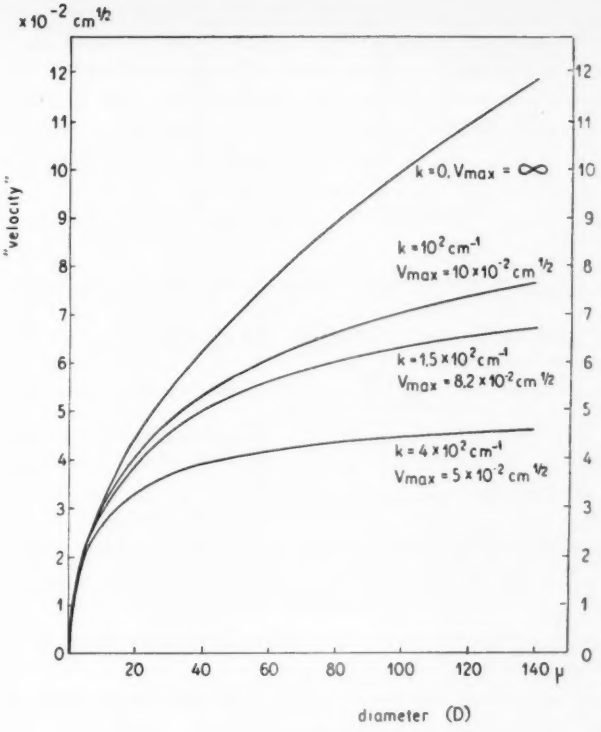

Fig. 5. "Propagation velocity" $\left(= \sqrt{\frac{D}{1 + \frac{r_e}{r_i}}} \right)$ as a function of fibre diameter (local circuit theory) assuming $\frac{r_e}{r_i} = k \cdot D$; r_e = external longitudinal resistance; r_i = internal longitudinal resistance; D = fibre diameter, and k a proportionality factor. The different curves correspond to different values of k.

(Fig. 6). Let these "rods" be separated by a small distance d, the interspace, containing a conducting fluid of specific resistance R_o. The impedance to transverse currents in a single layer of inactive fibres can be described by the equivalent circuit shown in Fig. 6. The internal impedance of the fibre and the leakage from the interspace to the fibre are omitted and the following values are assumed for the components of the equivalent system:

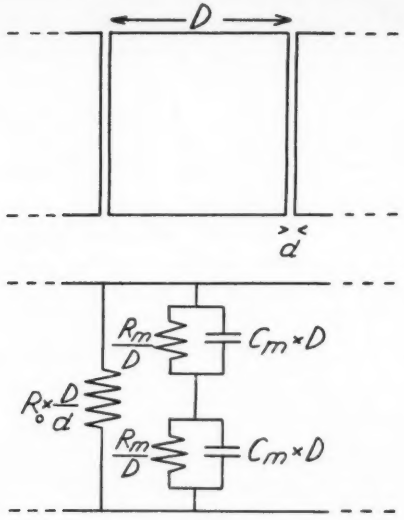

Fig. 6. *Above:* Cross section through a layer of fibres with extracellular spaces. The fibre is pictured as a square with side length D and the extracellular space as a layer of thickness d. *Below:* Equivalent circuit for the transverse impedance of a single layer of inactive fibres.

$R_o \cdot \frac{D}{d}$ = resistance per cm fibre length of a single interspace; R_o = specific resistance of extracellular fluid.

$\frac{R_m}{D}$ = transverse membrane resistance per cm fibre length of surface D, R_m = membrane resistance per square cm.

$C_m \cdot D$ = membrane capacitance per cm fibre length of surface D, C_m = membrane capacitance per square cm.

Internal fibre resistance and leakage from interspace to fibre are disregarded.

specific membrane resistance $R_m = 3{,}000\ \Omega\ cm^2$; specific membrane capacity $C_m = 5\ \mu F$ per cm^2; specific resistance of the extracellular fluid $R_o = 87\ \Omega$ cm (KATZ 1948); and mean fibre diameter $D = 50\ \mu$ (BUCHTHAL, GULD and ROSENFALCK 1955). For $\frac{d}{D}$ larger than $1.4 \cdot 10^{-3}$, the fibre resistance $\left(\frac{2}{D} R_m\right)$ exceeds the interspace resistance $\left(R_o \frac{D}{d}\right)$ by more than ten times and can be disregarded.

The steep deflection, *i. e.* the fastest change, of the action potentials lasts 100 to 300 μsec. (BUCHTHAL, GULD and ROSENFALCK 1954). Therefore, the membrane capacitance can be disregarded if the time constant of the transverse impedance of fibre plus interspace is below 100 μsec. (SOLMS, NASTUK and ALEXANDER 1953). The time constant τ is:

$$\tau = \left(\frac{R_o \cdot D}{d}\right) \cdot \left(\frac{C_m \cdot D}{2}\right) = 6 \cdot 10^{-7} \frac{D}{d} \quad \ldots\ldots\ldots \quad (4)$$

A time constant below 100 μsec. obtains with $\frac{d}{D}$ larger than $6 \cdot 10^{-3}$, corresponding to an extracellular volume of more than 1.2 per cent $\left(\text{extracellular volume} = 2\,\frac{d}{D}\right)$.

Thus, if the extracellular volume exceeds 1.2 per cent, the inactive fibres act as insulators and the transverse and longitudinal components of the action currents are distributed in the extracellular spaces. Therefore, the ratio between external and internal longitudinal resistance can be estimated in the following way: The internal longitudinal resistance per cm rod is $\frac{R_i}{D^2}$, where R_i represents the specific resistance of the myoplasm. The external longitudinal resistance is $c \cdot \frac{R_o}{4\,d\,(d+D)}$, where $\frac{R_o}{4\,d\,(d+D)}$ represents the resistance of the interspace immediately surrounding the fibre, and c a factor (less than 1 and independent of D and d) expressing that the currents spread over more than one interspace. With $R_i = 230\ \Omega$ cm and $R_o = 87\ \Omega$ cm (KATZ, 1948), the ratio between the external and the internal resistance is

$$\frac{r_e}{r_i} = \frac{c}{10.5} \cdot \frac{D}{d} \quad \ldots\ldots\ldots\ldots\ldots \quad (5).$$

The proportionality factor k defined by (2) can therefore be written

$$k = \frac{c}{10.5} \cdot \frac{1}{d} \quad \ldots\ldots\ldots\ldots\ldots \quad (6)$$

and a variation in propagation velocity similar to that found experimentally would obtain with:

$$\frac{l}{D_{min.}} = \frac{c}{10.5} \cdot \frac{l}{d} \quad \ldots\ldots\ldots\ldots\ldots\ldots (7).$$

Assuming $D_{min.} = 30\ \mu$ (BUCHTHAL, GULD and ROSENFALCK 1955), a 30 per cent variation in propagation velocity would obtain with $d = 2.9\ c$ (d in μ). The steep decrease in spike amplitude with increasing distance from the active fibre (to be published) justifies the assumption that the external resistance is mainly determined by the interspace immediately surrounding the fibre and therefore c is estimated to 0.5. Hence, to obtain a small variation in propagation velocity corresponding to that found in the present experiments, d must be equal to 1.5 μ. Assuming a mean diameter of 50 μ, this would entail an extracellular space smaller than $2 \cdot \frac{1.5}{50} = 6$ per cent. BOYLE, CONWAY, KANE and O'REILLY (1941) found an extracellular volume in muscle of 13 per cent. However, the extracellular volume need not be homogeneously distributed within the muscle and it might be less than 13 per cent within a primary fascicle. Therefore, it might very well be possible that the external resistance with fibres in situ actually diminishes considerably the variation of propagation velocity with diameter found for isolated fibres in Ringer's solution (HÅKANSSON 1954). A high external resistance and the reduced range of propagation velocities would facilitate the mechanism of mutual interaction between fibres of different diameters which previously was assumed to explain the narrow range of propagation velocities (BUCHTHAL, GULD and ROSENFALCK 1955). This interaction would further lower and equalize the propagation velocities.

For the different fibres in whole nerve, propagation velocity varies considerably with the fibre diameter (ERLANGER and GASSER 1937). This suggests that the external resistance within a nerve does not affect propagation.

Summary.

The innervation of the brachial biceps was found to be localised to a narrow band in the proximal third of the short head and in the distal third of the long head. Counts of terminal nerve endings showed that 67 per cent of all endings were situated within 10 per cent of the muscle length.

The action potential recorded with an electrode placed within the zone of innervation had an abrupt onset, and with two or more electrodes placed in the innervation zone near fibres of the same motor unit, the initial deflection of the action potential was simultaneous within 0.5 msec. This was seen up to electrode distances of 40 mm, along the longitudinal axis of the muscle, and indicates that the end plates of the same unit are scattered over this area. It excludes furthermore that propagation over the thin terminal nerve branches contributes to the action potential duration.

When care was taken to place the electrodes distal to the site of innervation and an identical threshold to electrical stimulation had ensured that the electrodes led off from the same muscle fibres, the propagation velocity of the spike of the action potential released by voluntary effort varied over a narrow range. The mean value was 4.7 m/sec. with a maximal variation of 1.3 m/sec. within the same muscle (36.5° C.). The small variation in velocity was unexpected in view of the threefold variation in diameter, but could be accounted for by the influence of the external resistance which was shown to be localised mainly in the interspaces between inactive fibres in situ.

The propagation velocity during voluntary effort exceeded the velocity of electrically activated fibres by 0.7 m/sec.

Differences in action potential duration during voluntary effort can mainly be attributed to the spatial dispersion of the site of innervation for the different fibres of the motor unit.

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Drinking, Antidiuresis and Milk Ejection from Electrical Stimulation within the Hypothalamus of the Goat.

By

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Electrical stimulation was recently shown to be a convenient, effective way to study hypothalamic polydipsia in the goat (ANDERSSON and McCANN 1955). When tested in one animal, stimulation at the same point where polydipsia was evoked produced a marked inhibition of water diuresis of neurohypophyseal type. As it seemed to be of interest to know if this would always be the case and to get a better knowledge of the extent of the area of the hypothalamus where stimulation could cause polydipsia, the experiments reported below have been undertaken.

Methods.

Sixteen fullgrown, female goats, most of them lactating, were used for the experiments. They were fed on hay and crushed oats, were offered water ad libitum and had free access to NaCl in the form of block salt.

For electrical stimulation HESS' technique (1932, 1949), somewhat modified for use in goats (ANDERSSON 1951) was used. The parameters of stimulation were 0.5 to 1.5 V and 50 c/s.

Parts of the experiments were filmed.

When recording urine flow the animals were placed in a modified Pavlov stand and catheterized with a Dowse's selfretaining catheter. In order to observe milk ejection the teats of lactating animals were cannulated during the experiments.

Sodium and potassium analyses were made using the EEL flame photometer and urine chloride was determined according to the Volhard method (PETERS and VAN SLYKE 1932). Creatinine in plasma and urine was measured as described by PEETERS (1942).

Histological technique: After killing the animals by decapitation, the heads were perfused with Bouin's fixative. After fixation in Bouin's and paraffin imbedding, the hypothalamus with attached pituitary was serially sectioned and stained by Gomori's chrom alum hematoxylin method (GOMORI 1941) as described by BARGMANN (1950). The localisation of the effective points of stimulation was in most cases facilitated by a small electrocoagulation made prior to killing the goats.

Results.

Electrical stimulation had an obvious polydipsic effect at 33 different points of stimulation in the hypothalamus of 9 goats. At 7 points this polydipsia was combined with inhibition of water diuresis and milk ejection. At one of these points only polydipsia and antidiuresis was observed since the animal was not lactating. The remaining points where polydipsia was evoked did not give rise to antidiuresis or milk ejection. Antidiuresis and milk ejection without obvious polydipsia were seen at 8 points of stimulation.

The region of the hypothalamus where polydipsia was observed in the absence of inhibition of water diuresis and milk ejection was located between Columna fornicis descendens and Tractus Vicq d'Azyr and extended from the dorsal into the ventral hypothalamus (Fig. 1, open circles). Lateral to the paraventricular nucleus and extending rostrally from the Columna fornicis descendens approximately to a transverse section through the anterior commissure, electrical stimulations resulted in polydipsia, inhibition of water diuresis and milk ejection (Fig. 1, black circles). Stimulations within or adjacent to the paraventricular nucleus have so far not caused polydipsia but did produce inhibition of water diuresis and milk ejection (Fig. 1, horizontal section 2, black half moons).

Similarly, stimulations within the supraoptic nucleus or in the supraoptico-hypophyseal tract caused, as expected, inhibition of water diuresis (HARRIS 1947) and milk ejection (ANDERSSON 1951, CROSS and HARRIS 1951), but here on no occasion was polydipsia seen as a result of the stimulations (Fig. 1, horizontal section 3, black half moons).

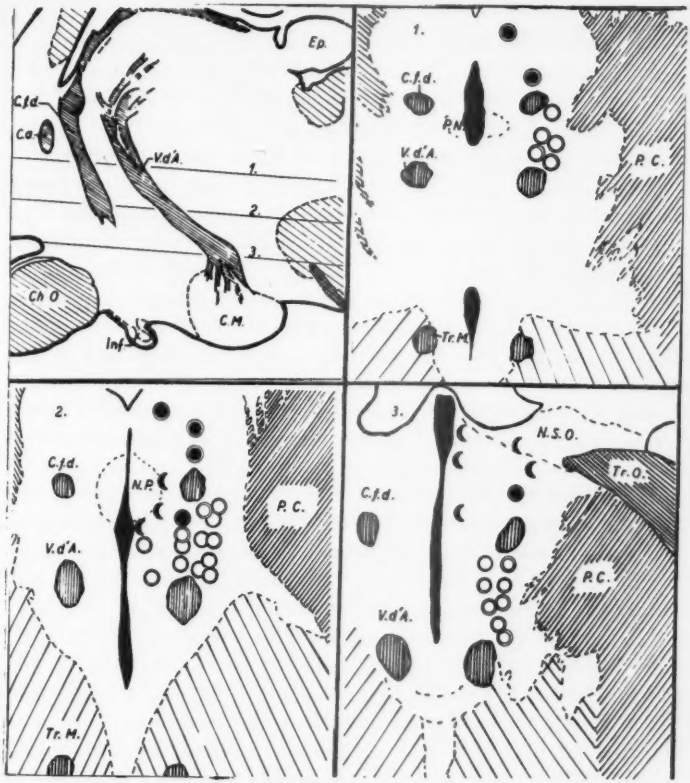

Fig. 1. *Points in the hypothalamus where electrical stimulation evoked drinking, antidiuresis and milk ejection.*

Diagrams of a sagittal section, and, corresponding to the lines labelled 1, 2 and 3, three horizontal sections (1, 2 and 3) through the hypothalamus of the goat.

Black circles: Points where electrical stimulation caused drinking, inhibition of water diuresis and milk ejection.

Open circles: Points where drinking was obtained in the absence of antidiuresis and milk ejection.

Black half moons: Points where stimulation gave inhibition of water diuresis and milk ejection, but no drinking.

C. A. = Commissura anterior.
C. f. d. = Columna fornicis descendens.
C. M. = Corpus mammillare.
Ch. O. = Chiasma opticum.
Ep. = Epiphysis.
Inf. = Infundibulum.
N. P. = Nucleus paraventricularis.
N. S. O. = Nucleus supraopticus.
P. C. = Pedunculus cerebri.
Tr. M. = Tractus Meynert.
V. d'A. = Tractus Vicq d'Azyr.

Details about stimulatory effects:

A) *Polydipsia.* The main features of polydipsia caused by electrical stimulations within the hypothalamus of the goat have been described earlier (ANDERSSON and McCANN 1955). Thus, enormous overhydration — up to 35 % of the body weight — accompanied by marked hemodilution and even hemolysis could be produced by prolonged stimulation at the positive points. The time of latency before the onset of drinking varied between 6 and 45 seconds and drinking usually continued without interruption during the stimulation and for 3 to 6 seconds thereafter.

Repeated short periods of stimulation often seemed to facilitate the drinking response as shown by a gradual shortening of the latency before the onset of drinking.

Although the animals usually found the water easily by themselves, restlessness caused by the stimulations occasionally interfered with an animal's ability to locate it. This restlessness was, however, notably absent when stimulating in that part of the "drinking area" lying rostral to the Columna fornicis descendens.

The goats were occasionally offered a mixture of urine and water during stimulation. They drank a weak water mixture but definitely refused a more concentrated solution.

It might be worth mentioning that the animals had to be quite alert if good drinking responses were to be obtained. If, for example, the placing of the electrodes had produced bleeding into the ventricle or similar damage causing an animal to become sluggish, which happened occasionally, stimulations obviously within the "drinking area" would not have any polydipsic effect.

B) *Inhibition of water diuresis and milk ejection.* After the animals had been sufficiently hydrated by electrically induced drinking or if necessary with water administered by stomach tube, the effect on the resulting water diuresis and on the milk flow of a 4 minute period of stimulation was evaluated. During these periods of stimulation the animals were also offered water, but were only allowed to drink, negligible amounts. When an obvious inhibition of water diuresis was seen, it always occurred together with milk ejection in lactating animals. The inhibition was of neurohypophyseal type and lasted for 45 minutes to one hour. During the period of inhibition there was a marked rise in urinary concentrations of Na, K and Cl, and in the specifik gravity of the urine, whereas the urinary excretion of electrolytes showed an inconstant

Table 1.

The effect on urinary sodium and chloride excretion of electrical stimulation at points where antidiuresis was produced.

Experiment	Goat	Initial Excretion meq./10 min.		% increase in excretion after stimulation	
		Sodium	Chloride	Sodium	Chloride
1.....	E	5.0 (2)[1]	3.7	30 (3)	— 10
2.....	K	3.8 (1)	2.9	130 (4)	100
3.....	M	4.6 (2)	2.9	80 (4)	110
4.....	M	0.75 (2)	0.17	10 (3)	240
5.....	O	11.6 (2)	7.4	0 (3)	0
6.....	O	10.4 (2)	4.6	— 30 (2)	— 10
7.....	P	5.4 (3)	2.7	70 (4)	70
8.....	P	9.0 (2)	3.5	40 (4)	70
9.....	B	0.66 (1)	1.00	30 (4)	0
Mean				40	67

[1] The figures in parenthesis give the number of 5 minute periods averaged. The numbers for chloride are the same as those indicated for sodium.

picture. There was generally an increase in urinary excretion of Na and Cl during the inhibition, but sometimes no obvious increase or even small decreases were seen (Table 1). Potassium showed a less consistent increase after stimulation. In most experiments the electrolyte excretion was not followed until augmented excretion had returned to the prestimulation value.

In one experiment where endogenous creatinine clearance was determined this showed no significant change during the period of inhibition except for the same dead space effect which could be observed for the electrolyte excretion.

Milk ejection usually commenced one to two minutes after the onset of stimulation, and, as it was only determined by the increased flow of milk from the cannulated teats, no conclusions as to the intensity of the ejection can be drawn from the experiments.

C) *Other effects of the stimulations.* In contrast to the inhibition of water diuresis observed when stimulating within the anterior part of the "drinking area", stimulations in its posterior extent could often cause a *temporary increase in urine flow.* The increase appeared during the period of stimulation and lasted during the first five minute period of urine collection thereafter.

Simple *licking* movements, licking the sides of the cage or

Fig. 2. Two pictures showing the appearance of a goat exhibiting the strange behavior seen occasionally during electrical stimulation in the hypothalamus, or normally when smelling urine or genital secretion. See text.

even at times marked licking of block salt were seen frequently in association with the drinking response, but once the animals found the water they drank continuously and no obvious licking

was seen. Licking movements were, however, only rarely observed when stimulating in the rostral part of the "drinking area" in front of the Columna fornicis descendens.

Very marked *panting*, clearly related to the stimulation, was observed in two animals on stimulation within a small region in the anterior, dorsal hypothalamus near the anterior commissure. Stimulation at the more ventral of these two points also caused drinking.

A striking, stereotyped pattern of behavior was seen in three experiments when stimulating at the posterior, lateral border of the more ventral part of the "drinking area". The same pattern of behavior could be induced by letting the animals smell goats' urine or genital secretions and is often seen in stallions in association with smelling a mare in heat. Thus, the animals would stand in the cage while simultaneously elevating the upper lip and the head and looking about as if they were smelling something of special interest (Fig. 2). Occasionally the tail was also raised.

Micturition and *defecation* were also seen from stimulation in the same region as reported previously (ANDERSSON 1951). *Feeding* and *extra-masticatory movements* were not seen within the "drinking area" except for one case where the point of stimulation was localized to its very lateral and caudal border.

Discussion.

The present results confirm and extend our previous finding that electrical stimulation of the hypothalamus can evoke drinking in goats (ANDERSSON and MCCANN 1955), and the area outlined by electrical stimulation agrees quite well with that previously described for osmotic stimulations causing polydipsia (ANDERSSON 1953, ANDERSSON and MCCANN 1955). Since a thorough exploration of the hypothalamus has now been made, the area outlined may represent the full extent of the hypothalamic region concerned in this response. The fact that stimulation within the hypothalamus can provoke polydipsia has recently been confirmed in another species by GREER (1955). He reports one rat in which electrical stimulation within the area previously described (ANDERSSON 1953, ANDERSSON and MCCANN 1955) caused polydipsia.

As drinking is so reproducible and can be forced to levels far

beyond physiological limits by electrical stimulation, this "drinking area" in the hypothalamus may contain the receptors normally responsible for the production of thirst. If this were the case, the complete ablation of it should abolish drinking. Consistent with this view are the reports by STEVENSON et al. (1950) of hypodipsia combined with dehydration after lesions in the ventromedian nuclei of the rat and by WITT et al. (1952) that, if the lesions of diabetes insipidus dogs are extended dorsally and laterally in the hypothalamus, thirst may be abolished. Similarly, preliminary experiments in dogs where an attempt has been made to ablate the "drinking area" without producing diabetes insipidus indicate that a partial or temporarily complete loss of thirst can be produced in the absence of diabetes insipidus (ANDERSSON and McCANN 1955 b).

MAYER in 1900 correlated thirst with an increase in osmotically active material in the extracellular fluid, and others have later shown that intravenous injections of hypertonic saline elicit thirst. Since the classical studies of VERNEY (1947), it has been generally accepted that an osmoreceptor mechanism localized to the hypothalamus controls the conservation of water in the kidney by regulating the activity of the neurohypophysis. WOLF (1950) has even postulated that the same or similar receptors regulate water intake on the basis of his osmometric analysis of thirst. Even if the receptors for thirst are localized to the portion of the hypothalamus outlined here, the physiological stimulus for their activity has not been determined. The osmotic stimuli used previously may, however, have preferentially activated "thirst receptors" in the hypothalamus (ANDERSSON and McCANN 1955).

If all presumed osmoreceptors in the hypothalamus have a dual function in the regulation of both water intake and output, one should except that their stimulation would induce both drinking and a release of antidiuretic hormone. Both drinking and inhibition of water diuresis were indeed found here when stimulating the rostral portion of the "drinking area". Thus, if osmoreceptors do subserve this dual function, they could be expected to lie here. Then the caudal portion of the "drinking area" where no antidiuresis was found might represent fibres from the receptors to the cortex or other effector areas in a similar manner as the paraventricular and supraoptic nuclei may represent effectors concerned in antidiuretic hormone release, since no drinking was obtained from stimulation in or close to these nuclei.

A second explanation of the data obtained here is that there are osmoreceptors of two kinds in the hypothalamus, one group concerned with water conservation by the kidneys and the other with water intake. The two groups would then be localized to two areas which overlap in the region where both drinking and antidiuresis have been obtained from stimulation.

Thirdly, the inhibition of water diuresis which has been obtained in the region where drinking could also be evoked may simply have been due to stimulation of fibres running from the paraventricular to the supraoptic nuclei and/or the neurohypophysis since stimulations in or adjacent to the paraventricular nucleus evoked inhibition of water diuresis. The data presented do not allow a decision between these three or other possible explanations.

The fact that antidiuresis could be obtained when stimulating in or adjacent to the paraventricular nucleus is at variance with HARRIS' (1947) finding in the rabbit that such stimuli do not cause inhibition of water diuresis.

The inhibition of water diuresis seen in the experiments was in all probability due to a release of antidiuretic hormone. The time course of the inhibition, the increased concentration of urinary electrolytes and the increased specific gravity of the urine during the period of inhibition were similar to that from injected pituitrin in the goat (ANDERSSON 1954). Furthermore, when determined, the excretion of endogenous creatinine was not significantly altered suggesting no gross change in glomerular filtration rate during the inhibition of water diuresis. The cause of the variability in the urinary excretion of Na and Cl seen after electrical stimulations causing antidiuresis (Table 1) is not known, but it might have been due to the release of varying amounts of antidiuretic hormone during the stimulations.

Milk ejection was always seen combined with antidiuresis, when both phenomena were studied. This is consistent with the hypothesis (ABEL 1930) that only one hormone is released from the neurohypophysis, or that both the antidiuretic and oxytocic hormones are released together. However, although a clear cut milk ejection can easily be seen by observing simple milk flow, no conclusions can be drawn as to the amount of oxytocic activity responsible for the effect. Milk ejection caused by electrical stimulation in the paraventricular nucleus has also been described by CROSS (1955) in the rabbit, but only after adrenalectomy.

Cross (1953) showed a peripheral blocking action of adrenaline on the response of the mammary gland to oxytocin in the rabbit and gives evidence that his stimulations near the paraventricular nucleus in the rabbit also activate the sympathetico-adrenal system, which in turn blocks the response of the mammary gland to oxytocin.

No obvious sign of an activation of the sympathetico-adrenal system has been observed here when stimulating adjacent to the paraventricular nucleus, which could account for the fact that milk ejection appeared in the intact goat. Stimulations somewhat more caudally in the posterior part of the "drinking area", however, often caused a transient increase in urine flow, which might have been due to release of adrenaline, as it usually augments diuresis in the goat (Andersson 1955).

It is well known that animals can show "salt hunger" when sodium chloride is lacking in their diet, and Richter (1941) has found that adrenalectomized rats will select saline solutions to counteract excessive renal loss of salt. It is possible, therefore, that the extreme licking, especially when the goats selected block salt, during stimulation might be an activation of a central mechanism for regulation of salt intake.

Of the other side effects seen during the stimulations reported here, the strange pattern of behavior shown in Fig. 2 seems to be of interest. As this behavior is seen normally in goats when smelling urine or their own genital secretion and is also frequently observed under similar circumstances in horses, this might be a manifestation of an olfactory sexual reflex.

Summary.

1. In a further study of polydipsia caused by electrical stimulation within the hypothalamus of the goat, drinking could be obtained at 33 points of stimulation in 9 animals. The localization of these points is given.

2. In the anterior part of the "drinking area", drinking evoked by stimulation was combined with antidiuresis and milk ejection (7 points of stimulation).

3. Stimulation in or adjacent to the paraventricular ur supraoptic nuclei or in the supraoptico-hypophyseal tract was followed by inhibition of water diuresis and milk ejection. No drinking was seen during these stimulations.

4. Notable side effects seen during the stimulations were: temporary increase of diuresis (in the posterior part of the "drinking area"), licking, panting (in a small region in the anterior and dorsal hypothalamus) and a strange behavioral pattern, which normally may be related to a "sexual" smell in the goat.

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